

STUDIES ON SOME FACTORS CONTROLLING  
PROSTAGLANDIN PRODUCTION BY THE  
GUINEA-PIG UTERUS

by

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Thesis presented for the degree of  
Doctor of Philosophy  
University of Edinburgh

1988



In accordance with the requirements of regulation 3.4.7 this thesis has been composed by myself and the work presented herein is my own.

Simon C. Riley



## ACKNOWLEDGEMENTS

I wish to thank the following for their contributions:

My supervisor, Dr. Norman Poyser for his encouragement and advice throughout this project and during the preparation of the thesis.

I was supported during my studies by a Medical Research Council Research Studentship.

Miss Lorna Marshall for her technical assistance.

Mrs Jean Hunter and the staff of the Animal House for the care of my animals.

Miss Audrey Kerr for typing this thesis.

My parents for their enthusiasm and support throughout my School and University studies which made all this possible.

Dr Gonzo and my friends for their patience and for humouring me during the writing stage of this project.

Finally, I wish to acknowledge Nambarrie tea, without which this thesis may never have been completed.

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate SIMON CHRISTOPHER RILEY  
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Title of Thesis Studies on some factors controlling prostaglandin production by the guinea-pig uterus

Prostaglandin (PG)  $F_{2\alpha}$  is the uterine luteolytic hormone in the guinea-pig and many other non-primate species. In the guinea-pig, oestradiol acting on a progesterone-primed uterus is the optimum stimulus for  $PGF_{2\alpha}$  production in vivo, apparently by the stimulation of phospholipase (PL)  $A_2$ , the rate limiting step in PG synthesis.

The output of  $PGF_{2\alpha}$ , but not the outputs of  $PGE_2$  and  $PGI_2$  (measured as its stable metabolite 6-keto- $PGF_{1\alpha}$ ), from the guinea-pig uterus superfused in vitro and from guinea-pig endometrium in culture were selectively stimulated between days 7 and 15 of the oestrous cycle. This output reflects  $PGF_{2\alpha}$  synthesis because  $PGF_{2\alpha}$  output from cultured endometrium was inhibited by the non-steroidal anti-inflammatory drug, indomethacin.

The output of  $PGF_{2\alpha}$  from the day 15 uterus superfused in vitro was stimulated by treatment with the calcium ionophore A23187.  $PGF_{2\alpha}$  output from day 15 endometrium in culture for three days was inhibited by removing extracellular  $Ca^{2+}$  and also by treatment with TMB-8 (an intracellular  $Ca^{2+}$  antagonist). Both extracellular and intracellular  $Ca^{2+}$  were necessary for the stimulation and maintenance of the high output of  $PGF_{2\alpha}$  from the endometrium at the end of the oestrous cycle. The calmodulin inhibitors trifluoperazine and W-7 also decreased  $PGF_{2\alpha}$  output from cultured day 15 endometrium, implicating calmodulin as a mediator in this  $Ca^{2+}$ -regulated control of  $PGF_{2\alpha}$  synthesis.

Cellular and secreted proteins were synthesised by day 7 and day 15 guinea-pig endometrium in culture. Endometrial protein synthesis was inhibited by treatment with protein and RNA synthesis inhibitors; these inhibitors also inhibited endometrial  $PGF_{2\alpha}$  output and  $PGF_{2\alpha}$  synthesizing capacity (a measure of tissue PGH synthase levels). The administration of actinomycin D into the uterine lumen on day 10 in vivo prevented the stimulation of uterine  $PGF_{2\alpha}$  output and endometrial protein synthesis observed on day 15. These findings suggest that protein synthesis is involved in the stimulation of endometrial  $PGF_{2\alpha}$  synthesis towards the end of the oestrous cycle. The effects of these protein and RNA synthesis inhibitors were not due to a rapid inhibitory action on PGH synthase, and levels of PGH synthase in the endometrium were not the rate limiting step in  $PGF_{2\alpha}$  synthesis by the guinea-pig uterus. Phorbol 12-myristate, 13-acetate (a stimulator of protein kinase C) and hydrocortisone had no effect on endometrial  $PGF_{2\alpha}$  output demonstrating that a lipocortin-like protein was not involved in the control of endometrial  $PGF_{2\alpha}$  synthesis.

Oestradiol (1-100ng/ml) and oxytocin had no effect on  $PGF_{2\alpha}$  output from day 7 or day 15 cultured endometrium. However, progesterone (10-100ng/ml) and oestradiol (1000ng/ml) inhibited endometrial  $PGF_{2\alpha}$  output and endometrial  $PGF_{2\alpha}$  synthesizing capacity. These ovarian steroids also inhibited the synthesis of secreted proteins by day 15 guinea-pig endometrium in culture.

It is concluded that in the guinea-pig oestradiol acting on a progesterone-primed uterus stimulates the synthesis of endometrial protein(s) that with  $Ca^{2+}$  mediates the increased release of precursor for the increase in uterine  $PGF_{2\alpha}$  synthesis towards the end of the oestrous cycle.

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## SECTION 1

### GENERAL INTRODUCTION

#### History

Fresh human semen was studied by Kurzrok and Lieb (1930) who showed that it would either inhibit or stimulate the motility of strips of human uterus in vitro. Subsequently, Goldblatt (1935) and von Euler (1934 and 1935) showed a marked stimulation of the contractile activity of smooth muscle by human seminal fluid. Von Euler (1935) showed similar stimulatory activity in the seminal fluid of the monkey, sheep, goat and in extracts of sheep seminal vesicles. All these extracts, which were soluble in lipid solvents and were of acidic character, caused the contraction of smooth muscle and lowered the arterial blood pressure in experimental animals. The term "prostaglandin" was coined by von Euler in 1935 for this unknown factor which was thought to originate from the prostate gland. However, the factor originates from the seminal vesicle and it may have been called "vesiglandin" if this had been realised. In 1939, von Euler proposed that prostaglandin (PG) was a lipid-soluble, fatty acid which possibly contained a double bond and a hydroxyl group.

Von Euler's findings that this biological activity was due to a new group of highly active, lipid-soluble, unsaturated fatty acids was confirmed by Bergstrom (1949), who also realised the presence of more than one saturated, hydroxy fatty acid in partially purified prostaglandin extracts. Bergstrom and Sjovall (1957) then reported the isolation of a prostaglandin factor (what was to be later termed PGF) in pure crystalline form. Subsequently, Bergstrom and Sjovall (1960a,b) were able to elucidate the structures of PGF and PGE using X-ray crystallography, and these structures of what are now known as PGF<sub>1 $\alpha$</sub>  and PGE<sub>1</sub> were later confirmed using mass spectrometry by

Bergstrom, Ryhage, Samuelsson and Sjovall (1963). These studies showed that prostaglandin was not a single compound but a group of similar substances. Since these initial studies many prostaglandins have been isolated and identified.

### Chemistry and nomenclature of prostaglandins

The prostaglandins are oxygenated, unsaturated hydroxy fatty acids with 20 carbon atoms (see Figure 1.a). They possess a five-membered ring to which two carbon chains are attached (The prostanoid skeleton). The 'upper' and 'lower' chains are denoted  $\alpha$  and  $\omega$  and they contain seven and eight carbon atoms, respectively. In nature, prostanic acid is not known to occur.

The correct chemical name for each PG can be derived from the structure of prostanic acid, but this produces very long names, so, for the majority of prostaglandins, a trivial nomenclature is employed which is briefly explained here (see Figure 1.a; Andersen, 1969).

(a) The letter designated to the respective PG describes the differences in the five-membered ring. However, thromboxanes possess an additional oxygen atom enclosed in the ring, and PGG<sub>2</sub> and PGH<sub>2</sub> have the same ring structure.

(b) The degree of unsaturation of the side chains is indicated by the subscript number after the letter. Thus, PGE<sub>2</sub> contains two double bonds in the side chains. Prostaglandins of the "1" series invariably have only one double bond in the trans $\Delta^{13-14}$  position. PGs of the "3" series have another double bond in the cis $\Delta^{17-18}$  position.

(c) The isomeric form of the hydroxyl group attached to carbon atom number 9 in PGs of the F series (i.e. whether it is below or above the plane of the line) is designated  $\alpha$  and  $\beta$ , respectively.



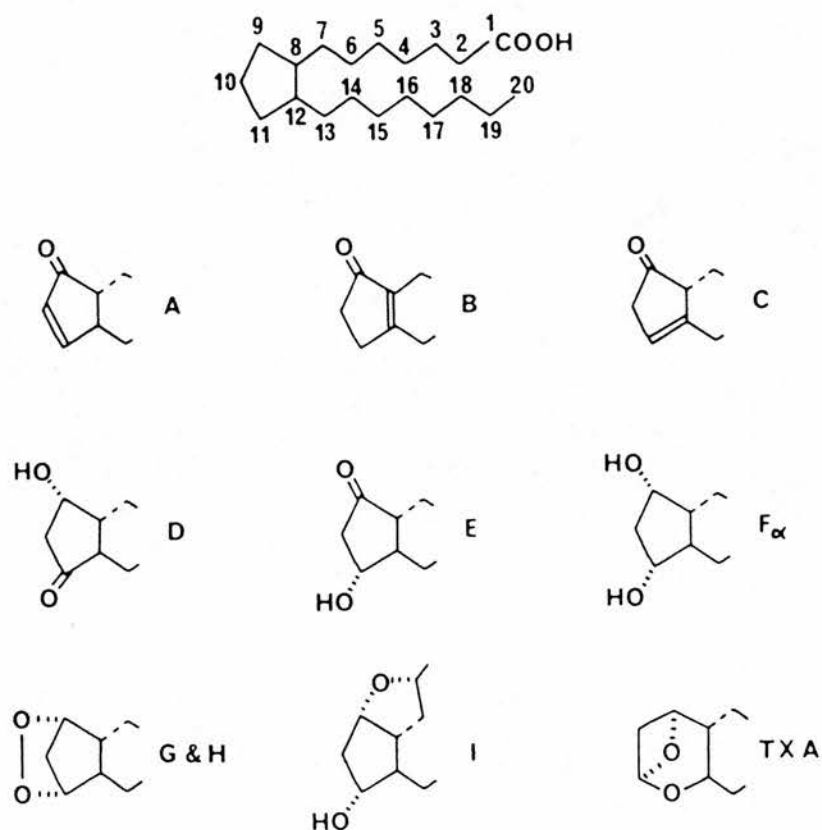


Figure 1.a

The prostanoic skeleton and the basic structures of the different groups of prostaglandins.

The "2" series PGs are the major PGs found in most biological systems, except for primate seminal fluid where "1" and "2" series PGs are found in equal quantities (Kelly, 1978). PGE<sub>2</sub> in acidic conditions dehydrates to form PGA<sub>2</sub>. PGA<sub>2</sub> can be enzymatically isomerised into PGC<sub>2</sub> followed by PGB<sub>2</sub> (Jones, Cammock and Horton, 1972). 19-Hydroxylated derivatives of PGE<sub>2</sub>, PGF<sub>2α</sub>, PGA<sub>2</sub> and PGB<sub>2</sub> have also been found.

### Biosynthesis

All naturally occurring PGs and thromboxanes are synthesised from three essential unsaturated fatty acids containing 20 carbon atoms, that are membrane bound in cells and are usually esterified in phospholipids. Arachidonic acid (5,8,11,14-all-cis-eicosatetraenoic acid) is the most important and is the source of the "2" series PGs. The other two fatty acid precursors are 8,11,14-all-cis-eicosatrienoic acid and 5,8,11,14,17-all-cis-eicosapentaenoic acid, and these are the precursors to the 1 and 3 series PGs, respectively. Arachidonic acid is also present in triglycerides and cholesterol esters, as well as phospholipids, but arachidonic acid bound to phospholipids represents the main source for PG synthesis and the release of arachidonic acid is the rate-limiting step in PG synthesis (Kunze and Vogt, 1971; Samuelsson, 1972; Hong and Levine, 1976; Flower and Blackwell, 1976; Vogt, 1978; Blackwell and Flower, 1979; Lands, 1979).

Prostaglandin biosynthesis occurs to a greater or lesser degree everywhere in the body. Essentially, all cell types except red blood cells possess the ability to synthesise PGs from fatty acids (Granstrom, 1981). The initial step in the formation of 2-series prostaglandins from arachidonic acid is the introduction of two molecules of oxygen at C-11 and C-15 by the enzyme prostaglandin H

synthase (PGH synthase), which requires haem and hydroperoxides for its activity (Lands, 1979). The continued presence of hydroperoxide appears to be required to maintain the reaction by a positive feedback system, and small amounts of hydroperoxide seem to be required to trigger synthesis (Lands, 1979). This PGH synthase enzyme has two activities, fatty acid cyclo-oxygenase (which converts arachidonic acid to PGG<sub>2</sub>) and prostaglandin hydroperoxidase (which converts PGG<sub>2</sub> to PGH<sub>2</sub>). The cyclo-oxygenase step is readily inhibited by non-steroidal anti-inflammatory drugs (e.g. indomethacin and aspirin). The endoperoxide PGH<sub>2</sub> can then be converted into a number of PGs, e.g. PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> and TXA<sub>2</sub>; the major ones synthesised depend upon the type of cell and the synthesizing enzymes it possesses (Samuelsson, Boldyue, Granstrom, Hamberg, Hammarstrom and Malmsten, 1978). PGI<sub>2</sub> is labile (half-life 3-5 min) and is rapidly hydrolysed to 6-keto-PGF<sub>1α</sub>. TXA<sub>2</sub> is also unstable (half-life of only 30 seconds) and is hydrolysed to TXB<sub>2</sub>.

Prostaglandins are metabolised at carbon-15 by oxidation of the secondary alcohol group into a ketone, which requires NAD<sup>+</sup> as a co-factor. These 15-keto-prostaglandins, especially of the F-series, may retain much of their biological activity of their parent molecule, but the subsequent reduction of the 13,14-double bond to 13,14-dihydro-15-keto-prostaglandins decreases the biological activity much more. These 13,14-dihydro-15-keto-PGs are the main circulating metabolites of PGF<sub>2α</sub> and PGE<sub>2</sub>, and there may be a 20-fold greater concentration of the metabolite in the circulation when compared to the parent molecule. Most tissues possess the ability to metabolise PGs, but often this is quite low. However, the lungs have a very high PG metabolising activity, and 95-99% of PGF<sub>2α</sub> and PGE<sub>2</sub> present in the circulation is metabolised by one passage through the lungs

(Ferreira and Vane, 1967). Furthermore, the metabolism of PGs is a two stage mechanism, involving an uptake step before the intracellular metabolism occurs due to  $\text{NAD}^+$ -dependent 15-hydroxyprostaglandin dehydrogenase.  $\text{PGF}_2\alpha$  and  $\text{PGE}_1$  exhibit a delay in their transit times through the pulmonary circulation, and Bito, Baroody and Reitz (1977) suggested that this is due to internalisation for metabolism. However,  $\text{PGI}_2$  and  $\text{PGD}_2$  are poor substrates for this uptake process. Therefore,  $\text{PGF}_2\alpha$  and  $\text{PGE}_2$  do not act as circulating systemic hormones while  $\text{PGI}_2$  and  $\text{PGD}_2$  could act as circulating hormones. The half-life of  $\text{TXA}_2$  is too short for it to be considered as a circulating hormone.

Prostaglandins are not stored in tissues, but are synthesised and released immediately (Piper and Vane, 1971), with the exception that PGs are stored in seminal fluid. The concentration of free arachidonic acid in tissues is low, so before PG biosynthesis can occur, arachidonic acid must first be released from some bound source. The primary source of arachidonic acid is phospholipids. Arachidonic acid is released by phospholipase  $\text{A}_2$  and intracellular phospholipase  $\text{A}$  activities have been found in almost all cells that have been examined (Van den Bosche, 1980). Prostaglandins and thromboxanes produced by tissues have many actions in the body, and the significance of prostaglandin production by the uterus has been investigated for many years, especially in relation to luteolysis.

#### Uterine Luteolytic Hormone

The importance of the uterus as an endocrine organ, that could determine the length of the oestrous cycle, was realised by Loeb in 1923. He showed that hysterectomy of the guinea-pig inhibited corpus luteal regression, and prolonged the oestrous cycle to between 60 and 120 days. Perceptively, Loeb in 1927 wrote "It is possible that the

uterus, in particular the mucosa, produces an internal secretion which exerts a specific abbreviating effect on the life of the corpus luteum". The theory that a uterine luteolytic hormone exists, which causes the regression of the corpus luteum, was thereby proposed.

Subsequently, the removal of the uterus has been shown to prolong luteal function in other species, including the cow (Wiltbank and Casida, 1956; Anderson, Neal and Melampy, 1962), pig (Spies, Zimmerman, Self and Casida, 1958), pseudopregnant rat (Bradbury, 1937), rabbit (Asdell and Hammond, 1933) and the sheep (Wiltbank and Casida, 1956). However, in other species, including the dog (Cheval, 1934), monkey (Burford and Diddle, 1936) and human (Jones and Telinde, 1961; Beavis, Brown and Smith, 1969) hysterectomy does not appear to affect the life-span of the corpus luteum and so the uterus exerts no affect on cyclic ovarian function.

In many species, the uterine horn exerts an effect only on the corpora lutea in the ipsilateral ovary, with no effect on the corpora lutea in the contra-lateral ovary. In hemi-hysterectomised animals, the luteolytic factor affects the corpora lutea only in the ovary adjacent to the retained uterine horn so normal luteal regression occurs in the ovary contra-lateral to the hemi-hysterectomy. This effect has been shown in the sheep (Inskeep and Butcher, 1966), guinea-pig (Bland and Donovan, 1966) and the pseudopregnant rat (Barley, Butcher and Inskeep, 1966), and the transfer of the luteolytic factor from the uterus to the ovary on the same side was thought to be via the vasculature. Bland and Donovan (1966) suggested that this apparently local activity of the uterine luteolytic hormone precluded the involvement of the pituitary in the luteal-ovarian relationship. In the pig, Anderson, Butcher and Melampy (1961) showed that destruction of the endometrial layer of the

uterus often prevented luteal regression. Subsequently, Butcher, Chu and Melampy (1962) chemically destroyed the endometrial layer of the guinea-pig uterus and this procedure prolonged the oestrous cycle. It was therefore proposed that the endometrium was the source of the uterine luteolytic hormone.

The uterine luteolytic hormone, for it to be defined as such, must cause luteal regression as determined by histological studies (structural luteolysis). It must also cause functional luteal regression, indicated by a decline in plasma progesterone levels (functional luteolysis), subsequent to its release from the uterus.

From the earliest studies up until 1969, there was little success in isolating and identifying the uterine luteolytic hormone. However, in 1969,  $\text{PGF}_{2\alpha}$  was discovered to exhibit the same action on the ovary as the uterine luteolytic hormone in the guinea-pig (Blatchley and Donovan, 1969), rat (Phariss and Wyngarden, 1969) and rabbit (Gutknecht, Cornette and Phariss, 1969). Blatchley and Donovan (1969) showed that the intra-peritoneal injection of  $\text{PGF}_{2\alpha}$  (0.5 mg) administered twice daily for seven days to guinea-pigs hysterectomised on day 4 or 5 of the cycle resulted in luteal regression. Phariss and Wyngarden (1969) infused  $\text{PGF}_{2\alpha}$  into the lumen of the pseudopregnant rat uterus on days 5 and 6 of pseudopregnancy, and the progesterone content of the ovaries of these animals decreased compared to saline-treated control animals. In this experiment,  $\text{PGF}_{2\alpha}$  caused morphological luteal regression, and also functional luteal regression which was reflected in the decline in ovarian progesterone concentrations.

Subsequently,  $\text{PGF}_{2\alpha}$  was shown to have a luteolytic effect in other non-primate mammals, including the sheep (McCracken, Glew and Scaramuzzi, 1970), cow (Liehr, Marion and Olsen, 1972; Lauderdale, 1972) and the pig (Gleeson, 1974).

Uterine venous blood concentrations of  $\text{PGF}_{2\alpha}$  were demonstrated to increase towards the end of the oestrous cycle, just before the occurrence of luteal regression, in the sheep (Bland, Horton and Poyser, 1971; McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972), pig (Gleeson and Thorburn, 1973; Gleeson, Thorburn and Cox, 1974), cow (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider and Thorburn, 1973) and guinea-pig (Blatchley, Donovan, Horton and Poyser, 1972). The uterine venous plasma concentrations of  $\text{PGF}_{2\alpha}$  increased from about day 11 of the oestrous cycle, although this timing was not absolute. Plasma  $\text{PGF}_{2\alpha}$  levels had increased significantly by day 13 and in all these species, luteal regression was generally complete by days 16 to 18. Uterine venous plasma levels of  $\text{PGF}_{2\alpha}$  were high throughout this period of luteal regression.

Bland and Donovan (1966) inserted glass beads into the lumen of the guinea-pig uterus on days 2 to 4 of the oestrous cycle, and this advanced the occurrence of luteal regression to between days 9 and 11 of the cycle. Subsequently, Blatchley, Donovan and Poyser (1976) recorded in these animals with intra-uterine beads an increase in utero-ovarian venous plasma concentrations of  $\text{PGF}_{2\alpha}$  by day 8, and which peaked on day 12 when utero-ovarian venous plasma progesterone levels were reduced. This demonstrated that premature luteal regression induced by intra-uterine beads is associated with the premature release of  $\text{PGF}_{2\alpha}$  from the uterus. A similar effect of

intra-uterine inserts on increasing uterine  $\text{PGF}_{2\alpha}$  production also occurred in the sheep (Spilman and Duby, 1972), rat and hamster (Saksena, Lau and Castracane, 1974).

Further evidence to demonstrate that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone includes active immunisation against  $\text{PGF}_{2\alpha}$ , where the antibodies produced probably neutralise  $\text{PGF}_{2\alpha}$  released from the uterus into the circulation. Horton and Poyser (1974a) actively immunised guinea-pigs against  $\text{PGF}_{2\alpha}$  and the oestrous cycles of animals with high anti- $\text{PGF}_{2\alpha}$  titres were more prolonged compared to animals with lower antibody titres. Immunisation against  $\text{PGF}_{2\alpha}$  also prevented luteal regression in sheep (Scaramuzzi, Baird, Wheeler and Land, 1973).

The prevention of luteal regression by inhibiting uterine  $\text{PGF}_{2\alpha}$  luteal synthesis is further evidence that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone. The inhibitor of PG synthesis, indomethacin, which inhibits the cyclo-oxygenase component of PGH synthase (Vane, 1971) was used in a slow release preparation placed in the lumen of the guinea-pig uterus (Horton and Poyser, 1973). This local administration of indomethacin greatly prolonged oestrous cycle length. Subsequently, Poyser and Horton (1975) demonstrated that this effect was due to the prevention of luteal regression as shown by the maintenance of high plasma progesterone levels.

To reiterate the evidence that  $\text{PGF}_{2\alpha}$  causes luteal regression:

- i. Administration of  $\text{PGF}_{2\alpha}$  causes luteal regression and mimics the luteolysin action of the uterine luteolysis in vivo.
- ii.  $\text{PGF}_{2\alpha}$  is present in the uterine vein in increasing amounts towards the end of the cycle, preceding luteolysis.
- iii. Stimulation of  $\text{PGF}_{2\alpha}$  output (e.g. by the insertion of intra-uterine devices or beads) causes premature luteal regression.



- iv. Immunisation against  $\text{PGF}_{2\alpha}$  prolongs the luteal lifespan.
- v. Inhibition of  $\text{PGF}_{2\alpha}$  synthesis by indomethacin also prolongs the luteal lifespan.

These results show that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone, but how is the  $\text{PGF}_{2\alpha}$  produced by the uterus transported to the ovary to exert its luteolytic effect ?

#### The transfer of $\text{PGF}_{2\alpha}$ from the uterus to the ovary

Bland and Donovan (1969) showed that ligation of the vascular tissue to the uterus and ovaries in guinea-pigs resulted in maintenance of the corpora lutea. However, ligation of the oviduct had no effect on luteal regression (Oxenreider and Day, 1967; Bland and Donovan, 1969). These findings implicated the vascular system as the means of transport of  $\text{PGF}_{2\alpha}$  from the uterus to the ovary. In the sheep, ligation experiments on the uterine vasculature showed that the luteolytic factor in uterine venous blood must pass into the ovarian vein for luteolysis to occur (McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972). The ovarian artery runs over the surface of the utero-ovarian vein. Barrett, Blockey, Brown, Cumming, Goding, Mole and Obst (1971) showed that separation of the ovarian artery from the utero-ovarian vein prevented corpus luteum regression, and they proposed a counter-current mechanism of transfer of  $\text{PGF}_{2\alpha}$  from the vein to the artery. Subsequently, McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson (1972) infused [ $^3\text{H}$ ]- $\text{PGF}_{2\alpha}$  into the sheep uterine vein, before its confluence with the utero-ovarian vein, on day 14 of the oestrous cycle. Simultaneously, blood samples were taken from the ovarian artery and the iliac artery, and a small increase in the amount of the radiolabel was measured in the blood from the ovarian artery after 20 min, but not in the iliac artery, and

the peak of radioactivity in the ovarian artery occurred after a further 20-30 min. This showed a time lag in the transfer of the [ $^3\text{H}$ ]-PGF $_{2\alpha}$  from the utero-ovarian vein to the ovarian artery. From these studies it was estimated that the amount of PGF $_{2\alpha}$  transferred by the counter-current transfer process was in excess of 5%. Horton and Poyser (1972) pointed out that the efficiency of transfer of PGF $_{2\alpha}$  must be quite low because if this counter-current mechanism of PGF $_{2\alpha}$  from vein to artery was too efficient, the levels of PGF $_{2\alpha}$  in the system would increase and remain high due to the inability of the local tissues to metabolise prostaglandins. Indeed, if the transfer process were 100% and the metabolism were nil, PGF $_{2\alpha}$  would pass through the ovary ad infinitum. The PGF $_{2\alpha}$  that is not retained by the counter-current mechanism could pass into the systemic circulation and be inactivated by the lungs (Ferreira and Vane, 1967). Subsequently, the estimated amounts of radiolabelled PGF $_{2\alpha}$  transferred from the uterine vein into the ovarian artery in the sheep were estimated by Land, Baird and Scaramuzzi (1976) to be 0.1 - 0.56%, and by Heap, Fleet and Hamon (1985) to be 0.3%. This counter-current mechanism has also been demonstrated in the cow (Hixon and Hansel, 1974).

The vasculature of the guinea-pig, rat, hamster and pig is so arranged to allow a counter-current mechanism similar to that proposed for the sheep (Del Campo and Ginther, 1972 and 1973). However, Egund and Carter (1974) could not find very close contacts between the ovarian artery and utero-ovarian vein in the guinea-pig. Furthermore, the uterine vasculature and the ovarian vasculature are independent in the rabbit (Hunter and Casida, 1967) and horse (Del Campo and Ginther, 1973), so a counter-current mechanism cannot explain this transfer of PGF $_{2\alpha}$  from the utero-ovarian vein into the ovarian artery in these two

species. Therefore, there may be species differences in the mode of transfer of  $\text{PGF}_{2\alpha}$  from uterus to ovary, or an alternative mechanism may exist. Hansel, Concannon and Lukaszewska (1973) suggested that part of the luteolytic factor may leave the uterus via the lymphatic system. Recently, a counter-current transfer system for  $\text{PGF}_{2\alpha}$  has been proposed in the pig (Kotwica, 1980) and sheep (Staples, Fleet and Heap, 1982), between the lymph vessels and the ovarian artery due to the proximity of these vessels. The concentration of  $\text{PGF}_{2\alpha}$  in uterine lymph was shown to be low during the first two-thirds of the oestrous cycle in the non-pregnant sheep, and then to increase after day 12 (Abdel Rahim, Bland and Poyser, 1983). Heap, Fleet and Hamon (1985) showed that  $[^3\text{H}]\text{-PGF}_{2\alpha}$  infused into a uterine lymph vessel was recovered from the ovarian blood in the sheep. Heap, Fleet and Hamon (1985) estimated that the efficiency of  $[^3\text{H}]\text{-PGF}_{2\alpha}$  transfer from a lymphatic vessel into ovarian blood (0.4%) was similar to that from a uterine vein (0.3%) into ovarian blood. Clearly, the lymphatic and vascular system possess the potential to transfer the luteolytic hormone to the ovarian vasculature of the sheep. In the sheep, Abdel Rahim, Bland and Poyser (1984) inserted a glass cannula into the uterine branch of the utero-ovarian vein of the sheep, and isolated the uterus and ovary from any other tissue contact. Luteal function was extended, and it was concluded that the transfer of  $\text{PGF}_{2\alpha}$  solely from uterine venous blood is not sufficient to cause luteolysis. The lymphatic system also appears necessary in the transfer of  $\text{PGF}_{2\alpha}$  to the ovary in the sheep (Abdel Rahim, Bland and Poyser, 1984). This alternative method of transfer of  $\text{PGF}_{2\alpha}$  via the lymphatic vessels may explain the anatomical differences that do not appear to allow a counter-current mechanism within the vascular system, for instance in the horse (Del Campo and Ginther, 1973) and the lymph may contribute

to the transfer of  $\text{PGF}_{2\alpha}$  to the ovary in other species that also process a counter-current vasculature system. Interestingly, Krzymowski, Czarnocki, Koziorowski, Kotwica and Stefanczyk-Krzymowska (1986) reported that the vasculature of the pig uterus exhibited a mechanism of  $[\text{}^3\text{H}]\text{-PGF}_{2\alpha}$  uptake by the mesometrium and its back transfer to the uterus. This mechanism was active when corpora lutea function was maintained, and strongly suppressed when luteal regression was occurring.

Transfer of  $\text{PGF}_{2\alpha}$  via the uterine lymphatic system as well as the uterine vascular system to the ovarian artery is the mechanism of transfer of  $\text{PGF}_{2\alpha}$  from the uterus to the ovary in the sheep enabling the uterus to exert its control on the function of the corpora luteum. It is also interesting to speculate whether a back-transfer system as reported in the pig, exists in the guinea-pig, sheep and other animals, and the degree of control this back transfer system exerts on luteal function.

The next section examines how the release of the uterine luteolytic hormone for transfer to the ovary to exert its effect on the corpus luteum is controlled and stimulated at the appropriate stage in the cycle.

#### The mechanism by which $\text{PGF}_{2\alpha}$ causes luteal regression

Prostaglandin  $\text{F}_{2\alpha}$  possesses potent vasoconstrictor properties (DuCharme, Weeks and Montgomery, 1968). It was proposed that  $\text{PGF}_{2\alpha}$  may cause reduced blood flow to the ovary, or more specifically to the corpus luteum, causing luteolysis due to anoxia (Pharriss and Wyngarden, 1969; Pharriss, Cornette and Gutnecht, 1970). Luteal tissue was shown to have a very high blood flow in sheep (Thorburn and Hales, 1972), with total ovarian blood flow greatest at times when

functional corpora lutea are present. Both luteal and ovarian blood flow rates were shown to decrease around the time of luteal regression (Niswender, Moor, Akbar, Nett and Diekman, 1975). Niswender, Reimers, Diekman and Nett (1976) concluded that blood flow to the corpus luteum may be important in the regulation of luteal function, including changes occurring during luteal regression. However, McCracken, Glew and Scaramuzzi (1970) and Baird (1974) could not show a decline in blood flow preceding the fall in progesterone secretion during luteal regression in the sheep. Subsequently, Einer-Jensen and McCracken (1977) observed that progesterone output decreased from the corpus luteum before the decrease in luteal blood flow. In the guinea-pig, Hossain, Lee, Clarke and O'Shea (1979) using radioactive microspheres demonstrated that ovarian and luteal blood flow were high at least until day 12 of the oestrous cycle. These results were in agreement with a study by Sjoquist, Bjellin and Carter (1977) in the guinea-pig. Ovarian and luteal blood flow fall rapidly and to a large extent during the period when progesterone secretion is declining towards the end of the oestrous cycle of the guinea-pig, although there was no evidence to suggest that the fall in luteal blood flow preceded the fall in plasma progesterone concentrations (Hossain, Lee, Clarke and O'Shea, 1979). O'Shea, Nightingale and Chamley (1977) suggested that this reduced luteal blood flow, occurring later when plasma progesterone levels are already in decline, may be caused by small luteal capillaries becoming blocked by cell debris, and not by a constrictor action of  $\text{PGF}_{2\alpha}$ . Clearly, whether  $\text{PGF}_{2\alpha}$  is or is not involved in the control of luteal blood flow, vasoconstriction is not the initial stimulus for luteal regression, but is only a secondary and possibly contributory effect.

Luteal regression is caused by  $\text{PGF}_{2\alpha}$  in the sheep, cow, pig, guinea-pig, pseudopregnant rat and many other non-primate mammalian species; in primates  $\text{PGF}_{2\alpha}$  causes luteal regression in monkeys, but not in women (see Horton and Poyser, 1976). However,  $\text{PGF}_{2\alpha}$  does not cause luteolysis early in the lifespan of the corpora lutea. Luteolysis is caused by  $\text{PGF}_{2\alpha}$  after day 5 in the cow, sheep, mare, and rat, after day 10 in the guinea-pig, and after day 12 in the pig. Clearly, there is a "developmental stage" early in the life of the corpus luteum which must occur before structural luteal regression can take place.

The presence of  $\text{PGF}_{2\alpha}$  binding sites (receptors) on the luteal cell membranes of the corpus luteum have been shown in the sheep (Powell, Hammerstrom and Samuelsson, 1974), cow (Rao, Carmon and Gorman, 1978), rat (Wright, Luborskymoore and Behrman, 1979) and the mare (Kimball and Wyngarden, 1977). In the cow, the binding of  $\text{PGF}_{2\alpha}$  to corpora lutea increases during the cycle due to an increase in the affinity of the binding sites for  $\text{PGF}_{2\alpha}$ ; maximum affinity occurs during luteal regression (Rao, Estergreen, Carmon and Mass, 1979). In the pseudopregnant rat, the binding of  $\text{PGF}_{2\alpha}$  to corpora lutea membranes increases to a maximum on day 7 and then slowly declines after day 9, which is before luteal regression occurs (Brambaifa and Schillinger, 1984). In the mare, there is no apparent correlation between the binding of  $\text{PGF}_{2\alpha}$  and the stage of the cycle (Kimball and Wyngarden, 1977). It seems that the affinity of binding sites (receptors) for  $\text{PGF}_{2\alpha}$ , or the number of receptors, or a combination of both factors may be involved in controlling luteal regression.

Luteinising hormone (LH) binds to its receptor and activates adenylate cyclase to cause an increase in cyclic adenosine 5'-monophosphate (cAMP) production, which stimulates luteal progesterone

formation and release. This LH-induced increase in cAMP production is prevented by  $\text{PGF}_{2\alpha}$  in rat corpora lutea within 15 min (Lahav, Freud and Lindner, 1976), and in sheep luteal cells (Fletcher and Niswender, 1982). LH binds to its binding sites (receptors) and in turn these bound receptors join together. These LH receptor aggregates appear necessary for the activation of adenylate cyclase and cAMP production (Luborsky, Slater and Behrman, 1984).  $\text{PGF}_{2\alpha}$  inhibits LH receptor movement within the membrane, so preventing LH receptor aggregation and the stimulation of adenylate cyclase (Luborsky, Slater and Behrman, 1984). Progesterone production is therefore reduced. In the rat (Behrman, Grinwich, Hichens and MacDonald, 1978), sheep (Diekman, O'Callaghan, Nett and Niswender, 1978) and pig (Barb, Kraeling, Rampacek and Pinkert, 1984),  $\text{PGF}_{2\alpha}$  appears to reduce LH binding to its receptor. However, when luteal cells receive a pulse of LH, binding of LH to these cells increases within 2h ('up regulation'), due to the appearance in the membrane of receptors that are not initially available to bind LH. Luborsky, Dorflinger, Wright and Behrman (1984) proposed that  $\text{PGF}_{2\alpha}$  prevents this 'up-regulation' of LH receptors. Therefore,  $\text{PGF}_{2\alpha}$  does not prevent LH binding to its receptor, but  $\text{PGF}_{2\alpha}$  decreases the overall binding of LH, due to it preventing the appearance of new LH receptors. Eventually, LH receptors are no longer present in the luteal cell membrane for the binding of LH. Are the effects of  $\text{PGF}_{2\alpha}$  in inhibiting the mobility of LH-binding sites in the membrane and in preventing LH binding to its receptor caused by the same action ?

Treatment of rat luteal cells with the calcium ionophore A23187, which increases the free intracellular  $\text{Ca}^{2+}$  concentration, causes the inhibition of LH-stimulated cAMP production and also inhibits directly LH-sensitive adenylate cyclase (Dorflinger, Albert, Williams and



Behrman, 1984).  $\text{PGF}_{2\alpha}$  stimulates the phosphatidylinositol (PI) cycle in rat luteal membranes (Raymond, Leung and Labrie, 1983), and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) produced from phosphatidylinositol 4,5-bisphosphate mobilises intracellular  $\text{Ca}^{2+}$  (Berridge, 1984). Therefore, in luteal cells  $\text{PGF}_{2\alpha}$  can release  $\text{Ca}^{2+}$  from intracellular stores by the stimulation of this second messenger system. Protein phosphorylation is also stimulated by increased turnover of the PI cycle, due to the stimulation of protein kinase C by diglyceride (Berridge, 1984).  $\text{PGF}_{2\alpha}$ -induced luteal regression is associated with increased membrane rigidification, and this change is calcium, calmodulin and protein dependent (Riley and Carlson, 1985). It was suggested that membrane proteins are phosphorylated by a calcium-dependent, calmodulin-modulated mechanism, causing membrane rigidification, and that this prevents the movement and appearance of LH receptors and hence LH binding to its receptor in the luteal membrane.

In the pig (Lemon and Moir, 1977) cow (Ursely and Leymarie, 1979), and sheep (Fitz, Mayan, Sawyer and Niswender, 1982), luteal cells are classified into two groups, namely the 'small' and 'large' cell types. Both groups of cells secrete progesterone, but the large luteal cell type secretes more progesterone than the small luteal cell type. However, there are apparently more small luteal cells present than large luteal cells, so the amount of progesterone secreted by each cell type is similar (Rodgers, O'Shea and Findlay, 1984). Progesterone secretion in small luteal cells is dependent on LH and cAMP. Dibutyryl-cAMP and agents that activate adenylate cyclase, for instance cholera toxin and forskolin stimulate progesterone production in small luteal cells (Hoyer, Fitz and Niswender, 1984). However, in large luteal cells, progesterone secretion is independent of LH and



cAMP (Niswender, Schwall, Fitz, Farin and Sawyer, 1985). Treatment of large luteal cells with cholera toxin or forskolin enhances the occupancy of cAMP-dependent protein kinase by cAMP (Niswender and Hoyer, 1985) but there is no resultant stimulation of progesterone production. This may be due to progesterone production by large luteal cells not being controlled by cAMP. Another possible explanation may be that progesterone secretion by large luteal cells cannot be further stimulated (Niswender, Schwall, Fitz, Farin and Sawyer, 1985). Furthermore, small luteal cells in sheep corpora lutea have few  $\text{PGF}_{2\alpha}$  receptors. The majority of  $\text{PGF}_{2\alpha}$  binding sites are found on large luteal cells (Fitz, Mayan, Sawyer and Niswender, 1982). Also, a greater proportion of small luteal cells are present early in the luteal lifespan, and large luteal cells increase in prevalence later in the oestrous cycle. This may explain the lack of effect of  $\text{PGF}_{2\alpha}$  early in the oestrous cycle (Fitz and Sawyer, 1982). Niswender, Schwall, Fitz, Farin and Sawyer (1985) proposed that LH might stimulate the differentiation of small luteal cells into large luteal cells. However, O'Shea, Rodgers and Wright (1986) examined the numbers and size of large and small ovine luteal cells. They discovered that there were fewer large luteal cells per unit volume on day 13 than on day 9, but that the mean volume of the large luteal cells was smaller on day 9 than on day 13. The total numbers of large luteal cells did not change, so small cells do not appear to differentiate into large luteal cells.

As the oestrous cycle progresses, the large luteal cells increase in volume and predominate over the small luteal cells. The large luteal cells possess  $\text{PGF}_{2\alpha}$  receptors and treatment with  $\text{PGF}_{2\alpha}$  results both in morphological changes in the large luteal cells, including extrusion of the cytoplasmic contents, and in functional

changes as the production of progesterone is inhibited (Fitz, Mock, Mayan and Niswender, 1984). Fitz, Mock, Mayan and Niswender (1984) proposed that 'PGF<sub>2α</sub> induces the large luteal cells to release a toxin which must accumulate to critical levels before exhibiting its inhibitory influence on large luteal cell morphology and function' and presumably this 'toxin' also acts on small luteal cells. Therefore, PGF<sub>2α</sub> causes both functional and structural luteolysis.

PGF<sub>2α</sub> may also directly inhibit cAMP-induced progesterone production by the corpus luteum of the rat (Jordan, 1981) and cow (Pate and Condon, 1984) by inhibiting the enzymes involved in progesterone synthesis, including cholesterol esterase (which converts cholesterol esters stored in lipid droplets into free cholesterol) and cholesterol ester synthetase (Torday, Jefcoate and First, 1980). In the hysterectomised guinea-pig, PGF<sub>2α</sub> causes a 50% inhibition of the conversion of pregnenolone to progesterone and reduces the activation of cholesterol esterase (but not of cholesterol ester synthetase) and of 3β-hydroxy-steroid-dehydrogenase (Dwyer and Church, 1979a, b). Furthermore, oestradiol has been suggested by Hixon, Gengenbach and Hansel (1975) to be required for luteolysis because, when sheep ovarian follicles are destroyed, PGF<sub>2α</sub> induced luteolysis is prevented.

PGF<sub>2α</sub> produced by the uterus is therefore capable of inducing functional and structural luteolysis in many non-primate species. But how is PGF<sub>2α</sub> synthesis by and release from the uterus stimulated in order to cause luteolysis at the appropriate stage in the oestrous cycle ?

### The physiological stimulus for PGF<sub>2α</sub> synthesis by the uterus

Oestrogen treatment of guinea-pigs on days 4 to 6 of the oestrous cycle caused premature luteal regression, and shortening of the oestrous cycle (Choudary and Greenwald, 1968; Bland and Donovan, 1968). Subsequently, by measuring the utero-ovarian plasma concentrations of PGF<sub>2α</sub>, Blatchley, Donovan, Horton and Poyser (1972) showed that oestradiol treatment on days 4 to 6 caused an early release of PGF<sub>2α</sub> from the guinea-pig uterus. They proposed that endogenous oestradiol released by the developing ovarian follicles after day 10 of the cycle was the physiological stimulus for uterine PGF<sub>2α</sub> synthesis.

In the guinea-pig, oestradiol concentrations in the ovarian venous plasma increased on day 10 of the oestrous cycle (Joshi, Watson and Labhsetwar, 1973), just before uterine PGF<sub>2α</sub> synthesis is stimulated as indicated by measuring PGF<sub>2α</sub> concentrations in the utero-ovarian plasma (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975). In the sheep, maximum plasma oestradiol concentrations occur between days 10 and 12 of the oestrous cycle, just before the stimulation of uterine PGF<sub>2α</sub> synthesis (Cox, Thorburn, Currie and Restall, 1974). It appears from this evidence that oestradiol may be the stimulus for uterine PGF<sub>2α</sub> synthesis.

To test this hypothesis, Blatchley and Poyser (1974) treated ovariectomised guinea-pigs with progesterone and/or oestradiol to mimic the ovarian steroid concentrations in intact animals, and to investigate the role of each steroid alone. Progesterone treatment produced a utero-ovarian venous plasma PGF<sub>2α</sub> concentration of  $3.7 \pm 0.5$  ng/ml ( $n = 5$ ), which is similar to concentrations between day 3 and day 10 of the oestrous cycle (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975). Oestradiol treatment

produced a utero-ovarian venous plasma concentration of  $\text{PGF}_{2\alpha}$  of  $12.0 \pm 5.3$  ng/ml, but only two of the six animals exhibited  $\text{PGF}_{2\alpha}$  concentrations that were higher than those receiving the progesterone treatment. However, animals which were treated with progesterone for 7 days followed by oestradiol and progesterone simultaneously for three days had increased uterine venous plasma concentrations of  $\text{PGF}_{2\alpha}$  of  $34.5 \pm 16.7$  ng/ml. Therefore, oestradiol acting on a progesterone-primed uterus was proposed as the optimum combination of the ovarian steroids for maximum stimulation of  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus (Blatchley and Poyser, 1974). Antonini, Turner and Pauerstein (1976) showed that  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus in vivo increased without any change on  $\text{PGE}_2$  output. Subsequently, Poyser (1983b) investigated the effect of oestradiol and progesterone treatment of ovariectomised guinea-pigs on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (the stable metabolite of  $\text{PGI}_2$ ) from guinea-pig uterine horns superfused in vitro. The outputs of all three PGs from uteri of ovariectomised guinea-pigs were low (0.2 ng/min/uterine horn). Progesterone treatment had no significant effect on PG output. Oestradiol treatment significantly stimulated  $\text{PGF}_{2\alpha}$  output 7-fold, but only increased  $\text{PGE}_2$  output 1.7-fold and 6-keto- $\text{PGF}_{1\alpha}$  output 2.9-fold. However, progesterone priming of the uterus followed by oestradiol treatment caused a further 2-fold increase in  $\text{PGF}_{2\alpha}$  output, but with no significant action on the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , when compared to the PG outputs from uteri removed from oestradiol-treated guinea-pigs (Poyser, 1983b). This study showed that oestradiol acting on a progesterone-primed uterus caused the relatively specific stimulation of uterine  $\text{PGF}_{2\alpha}$  output that is seen towards the end of the oestrous cycle of the guinea-pig.

In ovariectomised sheep, exogenous progesterone treatment for seven days significantly increased uterine venous plasma  $\text{PGF}_{2\alpha}$  concentrations (Scaramuzzi, Baird, Boyle, Land and Wheeler, 1977). However, progesterone treatment for fourteen days caused a decrease in  $\text{PGF}_{2\alpha}$  output from the sheep uterus compared to the output after seven days of treatment. Ottobre, Lewis, Thayne and Inskeep (1980) showed that early treatment of sheep with progesterone on days 0 and 1 of the cycle advanced the time of occurrence of the initial increases in uterine  $\text{PGF}_{2\alpha}$  output to day 8 compared to day 12 in control animals. From these findings, they proposed that a period of progesterone secretion controls the timing of the initial rises in uterine  $\text{PGF}_{2\alpha}$  output. McCracken (1980) infused ewes with progesterone for ten days starting immediately after oestrus, and recorded a stimulation of uterine  $\text{PGF}_{2\alpha}$  output in peaks of low magnitude on days 9 or 10 of treatment. Also, Lawson and Cahill (1983) demonstrated that the preparation of the uterus for pregnancy was advanced by early progesterone treatment, allowing successful embryo transfer of day 10 embryos on day 6 of progesterone treatment.

The output of  $\text{PGF}_{2\alpha}$  from the sheep uterus was increased by oestradiol treatment after several days of exogenous or endogenous progesterone treatment (Scaramuzzi, Baird, Boyle, Land and Wheeler, 1977; Barcikowski, Carlson, Wilson and McCracken, 1974). In the sheep uterus autotransplanted in the neck and infused with oestradiol in vivo,  $\text{PGF}_{2\alpha}$  output was stimulated after 60-90 min (Barcikowski, Carlson, Wilson and McCracken, 1974). However, there is still some controversy concerning the exact role of the ovarian steroids in the stimulus of uterine  $\text{PGF}_{2\alpha}$  output from the sheep, because Baird, Land, Scaramuzzi and Wheeler (1976) and Ottobre, Lewis, Thayne and Inskeep (1980) found no relationship between the initial rises in  $\text{PGF}_{2\alpha}$  output

and the increases in oestradiol levels in venous plasma. Furthermore, when follicles in the sheep ovary were destroyed to remove the main source of oestradiol, luteal regression was only delayed (Warren, Hawk and Bolt, 1973) and antisera to oestradiol did not delay luteal regression in the sheep (Fairclough, Smith and Peterson, 1976). Therefore, a rise in plasma oestradiol concentrations may not be necessary for the stimulation of  $\text{PGF}_{2\alpha}$  by the sheep uterus to cause luteal regression.

Kindahl, Lindell and Edquist (1981) showed in heifers that progesterone implants placed on day 12, just after the increase in uterine  $\text{PGF}_{2\alpha}$  output had begun, caused the maintenance of  $\text{PGF}_{2\alpha}$  output until the implants were removed. After removal (on day 26), plasma progesterone concentrations dropped and the animals ovulated two days later. Therefore, in vivo progesterone appears to play an important role in the control of the duration of uterine  $\text{PGF}_{2\alpha}$  synthesis.

Progesterone treatment in vitro inhibited  $\text{PGF}_{2\alpha}$  output from both proliferative and secretory human endometrium in organ culture (Cane and Villee, 1975; Abel and Baird, 1980), although the results of Tsang and Ooi (1982) only showed an inhibitory effect of progesterone on human proliferative endometrium in organ culture. Schatz, Markiewicz, Barg and Gurpide (1985) showed an inhibitory effect of progesterone on both human proliferative and secretory endometrium in organ culture, but the inhibitory effect was more marked on proliferative endometrium. Oestradiol treatment of human endometrium in organ culture exhibited a stimulatory effect, that was overcome by progesterone treatment (Abel and Baird, 1980; Schatz, Markiewicz, Barg and Gurpide, 1985). Leaver and Seawright (1982) showed progesterone treatment inhibited  $\text{PGF}_{2\alpha}$  output from both day 7 and day 15 guinea-pig endometrium in organ culture (days of low and high  $\text{PGF}_{2\alpha}$

output, respectively). The inhibitory effect occurred on day 2 of culture after a preincubation period of one day when no treatment was administered. Furthermore, oestradiol treatment significantly stimulated  $\text{PGF}_{2\alpha}$  output, an effect that was greater on day 7 than day 15 (Leaver and Seawright, 1982).

However, when the guinea-pig uterus was superfused in vitro and treated with oestradiol or progesterone, no change in  $\text{PGF}_{2\alpha}$  output was observed (Poyser and Brydon, 1983). This finding was confirmed using the same preparation by Poyser (1987b). There is evidently some controversy as to the in vitro effects of oestradiol and progesterone treatment when comparing the actions of these steroids on PG production by superfused uteri and cultured endometrium of the guinea-pig. The effect of these hormones must be further examined not only on  $\text{PGF}_{2\alpha}$  output, but also on  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs. Nevertheless, the evidence indicates that, in vivo, oestradiol acting on a progesterone-primed uterus is the physiological stimulus for increasing  $\text{PGF}_{2\alpha}$  synthesis and release from the guinea-pig uterus.

The infusion of oxytocin into the uterine arterial supply of the sheep stimulated  $\text{PGF}_{2\alpha}$  release from the uterus after treatment on day 14 of the oestrous cycle (Roberts, Barcikowski, Wilson, Skarnes and McCracken, 1975). Oxytocin has also been shown to stimulate release of  $\text{PGF}_{2\alpha}$  in the rat (Campos, Liggins and Seamark, 1980) and the rabbit (Small, Gavagan and Roberts, 1978). Oxytocin from the ovary is also necessary for increasing uterine  $\text{PGF}_{2\alpha}$  output in the goat (Cooke and Homeida, 1982; Cooke and Homeida, 1985) and probably the cow (Wathes, Swann, Birkett, Porter and Pickering, 1983). In the sheep, immunisation against oxytocin delays luteal regression (Sheldrick, Mitchell and Flint, 1980), but the endometrium only becomes responsive to oxytocin after plasma progesterone levels have started to decrease



(Roberts, McCracken, Gavagan and Soloff, 1976; Sheldrick and Flint, 1985) because progesterone seems to prevent the increase in oxytocin receptor numbers that is induced by oestradiol. Therefore, in the sheep, oestradiol acting on a progesterone primed uterus causes an initial increase in endometrial  $\text{PGF}_{2\alpha}$  output. The endometrial responsiveness to oxytocin only occurs after plasma progesterone concentration have started to decline, so oxytocin is involved in the maintenance of uterine  $\text{PGF}_{2\alpha}$  output in the sheep.

In the guinea-pig, there are conflicting reports concerning the role of oxytocin in the stimulation of uterine  $\text{PGF}_{2\alpha}$  output. Leaver and Seawright (1982) reported that oxytocin stimulated  $\text{PGF}_{2\alpha}$  output from day 15 guinea-pig endometrium in culture. However, Poyser and Brydon (1983) could find no stimulatory effect of oxytocin on  $\text{PGF}_{2\alpha}$  output from either the day 7 or day 15 guinea-pig uterus superfused in vitro. Furthermore, Donovan (1961) found no effect of oxytocin on oestrous cycle length when injected into the guinea-pig. The role of oxytocin in the control of uterine  $\text{PGF}_{2\alpha}$  synthesis and release appears to exhibit species differences. Also, this report by Leaver and Seawright (1982) of oxytocin stimulating  $\text{PGF}_{2\alpha}$  output in the cultured guinea-pig endometrium must be re-examined.

#### The pattern of release of $\text{PGF}_{2\alpha}$ output by the uterus

The output of  $\text{PGF}_{2\alpha}$  from the uterus occurs in pulsatile surges in the sheep (McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972; Thorburn, Cox, Currie, Restall and Schneider, 1972), cow (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider and Thorburn, 1973) and pig (Gleeson and Thorburn, 1973; Gleeson, Thorburn and Cox, 1974). To obtain the precise pattern of  $\text{PGF}_{2\alpha}$  release, samples of plasma must be



collected very frequently. Kindahl, Lindell and Edquist (1981) collected hourly plasma samples from the jugular vein of heifers, and measured the concentration of the main circulatory metabolite of  $\text{PGF}_{2\alpha}$  (13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$ ). The  $\text{PGF}_{2\alpha}$  metabolite showed a complex pattern of pulses of 1-5h duration during a 2-3 day period. Plasma PG metabolite levels increased prior to the decrease in plasma progesterone levels (Kindahl, Lindell and Edquist, 1981). Therefore,  $\text{PGF}_{2\alpha}$  appeared to initiate luteolysis. Kindahl, Lindell and Edquist (1981) observed a relationship between the plasma concentrations of the  $\text{PGF}_{2\alpha}$  metabolite and progesterone, because after each  $\text{PGF}_{2\alpha}$  metabolite peak occurred, the plasma progesterone concentration decreased in a stepwise pattern. The inhibitory action of  $\text{PGF}_{2\alpha}$  on progesterone production was very rapid (McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972).

Infusions of  $\text{PGF}_{2\alpha}$  were given directly into the arterial supply of the ovary autotransplanted to the neck in sheep (Schramm, Bovaird, Glew, Schramm and McCracken, 1983). It was discovered that four separate, hour-long infusions of  $\text{PGF}_{2\alpha}$  (0.04 - 0.08  $\mu\text{g}/\text{ml}$ ) given over 19h caused permanent luteal regression in only one of four animals, but the addition of a fifth and similar infusion of  $\text{PGF}_{2\alpha}$  (5 doses in 25h) caused permanent luteal regression in four out of four animals. A single daily infusion of  $\text{PGF}_{2\alpha}$  for four consecutive days caused a temporary fall in plasma progesterone levels after each daily "pulse" followed by a recovery. Permanent corpus luteal regression did not occur with this daily, long term pulsatile administration of  $\text{PGF}_{2\alpha}$ . A relatively short pulse frequency of  $\text{PGF}_{2\alpha}$  administration is therefore required in order to cause luteal regression in sheep. These ultra-low amounts of  $\text{PGF}_{2\alpha}$  administered in a pulsatile manner were proposed to be comparable with the quantitative estimates of the

amounts of  $\text{PGF}_{2\alpha}$  exchanged by the counter-current vascular system in the sheep (McCracken, Schramm and Okulicz, 1984), and were approximately one-fortieth of the amounts of  $\text{PGF}_{2\alpha}$  ( $2.5\mu\text{g/ml}$ ) required to cause luteolysis when given as a continuous infusion for 6-19h into the sheep ovarian artery (McCracken, Barcikowski, Carlson, Green and Samuelsson, 1973).

To date, it has proved impossible to examine the pattern of  $\text{PGF}_{2\alpha}$  release in the smaller animals (e.g. guinea-pig and rat) due to the difficulties in sampling. It is not known whether this pulsatile pattern of  $\text{PGF}_{2\alpha}$  release is a feature of the control of luteolysis in these smaller animals.

#### Mechanisms by which steroid hormones control uterine $\text{PGF}_{2\alpha}$ synthesis

##### 1. Oestradiol

The action of oestradiol in stimulating  $\text{PGF}_{2\alpha}$  synthesis may be due to its effect on controlling protein synthesis. Recently, there has been some controversy as to the mechanism by which oestradiol binds to its receptor. Prior to 1984, the model proposed by Jensen, Suzuki, Kawashima, Stumpf, Jungblat and DeSombre (1968) was generally accepted. In this two-step model, oestradiol diffuses into the cytoplasm where it binds to the oestrogen receptor. The oestrogen receptor was thought to be a cytosolic protein because it is recovered in the supernatant after homogenisation. The oestrogen receptor - oestradiol complex becomes activated and translocates to the nucleus where it binds to chromatin and induces specific mRNA synthesis. Szego (1974) proposed a model where oestradiol is captured by plasma membrane associated oestrogen receptors and the oestrogen receptor - oestradiol complexes are then internalised by pinocytosis and

transferred to the nucleus. However, little conclusive evidence has been found showing membrane bound oestrogen-receptors using recent techniques (Nelson, Clarke and Murphy, 1986).

Gorski, Welshons and Sakai (1984) proposed "the nuclear model of oestrogen receptor action", because immunocytochemical (King and Green, 1984) and enucleation experiments (Welshons, Lieberman and Gorski, 1984) suggested that the oestrogen receptor is situated in the nucleus, and that the binding of oestradiol to the oestrogen receptor and the activation of the oestrogen receptor - oestradiol complex occurs within the nucleus. Cytosolic oestrogen receptors were thought to be an extraction artifact. All the reports agreed that free oestrogen receptors have a low affinity for the nucleus, and that their dissociation rates were rapid relative to those of hormone-receptor complexes. Due to the high concentration of DNA in mammalian nuclei and due to receptors binding to genomic sites, a distribution of oestrogen receptors that is predominantly in the nucleus in intact cells may be expected, even for hormone-free receptors with a low affinity for the nucleus (Yamamoto, 1985). It may also be expected that a small fraction of receptors are transiently cytoplasmic or associated with the membrane and are available to bind hormone (Yamamoto, 1985). Nelson, Clarke and Murphy (1984) examined the techniques used and suggested that none of the models could be discarded until unequivocal evidence is produced in support of a subcellular location of the oestrogen receptor at all times.

In the rat, early responses to oestradiol occur within a few hours of binding to the oestrogen receptor. These include the synthesis of specific proteins, for instance oestrogen-induced uterine protein (IP), and the synthesis of a 49 kDa protein within 2h (Kumar,

O'Connor, Seeger, Beach and Dickerman, 1983). Komm, Rusling and Lyttle (1986) demonstrated increased production of two proteins (115 and 65 kDa) within 6h of a single injection of oestradiol. Similarly, oestradiol increased the activity of the enzymes ornithine carboxylase in the rat uterus (Rourke, Kendra and Katzenellenbogen, 1984), and of hydrolase (Katz, Finlay, Tom and Levitz, 1984) and arginine esterpeptidase in the mouse uterus (Henrikson, Jazin and Dickerman, 1987).

French and Casida (1973) demonstrated the importance of protein synthesis in the control of  $\text{PGF}_{2\alpha}$  output from the uterus. The administration of actinomycin D ( $500\mu\text{g}$ ) into the uterine lumen of the sheep on day 11 of the oestrous cycle prevented luteal regression. Furthermore, when actinomycin D treatment was administered only to one uterine horn, and transfer via the lumen to the other horn was prevented, luteal regression occurred only in the ovary ipsilateral to the non-treated horn. Therefore, the stimulation of DNA-dependent RNA synthesis, which is inhibited by actinomycin D, was necessary for the stimulation of the production of the luteolytic hormone from the sheep uterus. Castracane and Jordan (1976) found that actinomycin D administered systemically did not prevent  $\text{PGF}_{2\alpha}$  release from the rat uterus. Subsequently, Poyser (1979) demonstrated in the guinea-pig that the systemic administration of actinomycin D had no effect on luteal lifespan and by inference, had no effect on uterine  $\text{PGF}_{2\alpha}$  release. However, Poyser (1979) also demonstrated that intra-uterine administration of actinomycin D ( $10\mu\text{g}/\text{uterine horn}$ ) on day 10 increased oestrous cycle length in the guinea-pig. Plasma progesterone concentrations remained high during these extended cycles, presumably due to an increase in luteal life-span.  $\text{PGF}_{2\alpha}$  production by uterine homogenates in vitro was decreased on day 15

after intra-uterine actinomycin D treatment, and it was proposed that actinomycin D prevented the increase in uterine PGH synthase levels that occurs towards the end of the cycle. These effects of actinomycin D were not overcome by exogenous oestradiol treatment (Poyser, 1979). However, the studies by Poyser (1979) and by French and Casida (1973) did not demonstrate, by direct measurement, that intra-uterine actinomycin D treatment on day 10 or day 11 in vivo inhibits  $\text{PGF}_{2\alpha}$  output from the uterus of the guinea-pig and sheep on day 15. Therefore, further studies investigating the effects of protein synthesis inhibitors on  $\text{PGF}_{2\alpha}$  output from the uterus need to be performed.

## 2. Progesterone and glucocorticoids

$\text{PGF}_{2\alpha}$  concentrations in the utero-ovarian venous plasma obtained from ovariectomised guinea-pigs treated with progesterone were similar to the values observed in the utero-ovarian plasma of guinea-pigs on days 3-10 of the oestrous cycle (Blatchley and Poyser, 1974). However, progesterone was found to potentiate the stimulatory action of oestradiol on uterine  $\text{PGF}_{2\alpha}$  production in ovariectomised guinea-pigs. Consequently, how does progesterone act to exert this priming effect ? Also, glucocorticoids have been shown to alter PG production in a variety of tissues (Hirata, Schiffmann, Venkatasubramanian, Salomon and Axelrod, 1980) and their mode of action is also examined here.

In the GH<sub>3</sub> cell line derived from rat pituitary tumour and which exhibit apparently normal steroid receptor systems (in that the cells respond to glucocorticoid, oestrogen and progesterone), Welshons, Krummel and Gorski (1985) have found only a low level of receptors for all three steroids in the cytoplasmic fraction. The majority of the

unoccupied glucocorticoid and progesterone receptors are primarily located in the nucleus, like the oestrogen receptors. Philibert and Raynaud (1974) have reported a progesterone-specific receptor in immature guinea-pig uteri. An autoradiographic study by Sar and Stumpf (1974) demonstrated [ $^3\text{H}$ ]-progesterone binding to guinea-pig uteri. Subsequently, an immunohistochemical study using monoclonal antibodies, by Perrot-Applanat, Logeat, Groyer-Ricard and Milgrom (1985), has demonstrated that the progesterone receptors were confined to the nucleus of all cell types in the endometrium and myometrium of mature guinea-pigs. From other evidence however, the main site of the glucocorticoid receptor however has not been definitely localised to the nucleus (King, 1987).

Glucocorticoids bind to a specific domain on the glucocorticoid receptor (Carlstedt-Duke, Okret, Wrange and Gustafsson, 1982) and both glucocorticoids and progesterone-receptor complexes stimulate transcription and hence protein synthesis (Parker, 1983). Yamamoto (1985) proposed that the properties of these two steroid receptors and oestradiol receptors are similar. However, it is not known whether progesterone potentiates the stimulatory action of oestradiol on uterine  $\text{PGF}_{2\alpha}$  production through an effect mediated by it combining with its own nuclear receptor.

Glucocorticoid hormones are thought to exert their anti-inflammatory actions by increasing the production of proteins that inhibit phospholipase  $\text{A}_2$  activity, thus preventing the release of arachidonic acid for PG, TX and leukotriene synthesis. Inhibition of phospholipase  $\text{A}_2$  <sup>( $\text{PLA}_2$ )</sup> by glucocorticoid-stimulated proteins has been identified in rabbit neutrophils (Hirata, Schiffman, Venkatasubramanian, Salomon and Axelrod, 1980), rat peritoneal macrophages (Blackwell, Carnuccio, DiRosa, Flower, Parente and

Persico, 1980), guinea-pig lung perfusates (Blackwell and Flower, 1979) and rat renomedullary interstitial cells (Rothhut, Russo-Marie, Wood, DiRosa and Flower, 1983). These antiphospholipase proteins present in different cell types are immunologically and biochemically related, if not identical, and are collectively termed lipocortins (DiRosa, Flower, Hirata, Parente and Russo-Marie, 1984). Three different peptides have been identified with molecular weights ranging from 15,000 to 60,000 Daltons and monoclonal antibodies raised against lipocortin can react with all three types (Hirata, Notsu, Iwara, Parente, DiRosa and Flower, 1982). Indeed, the amino acid sequence of human lipocortin has been elucidated (Wallner, Mattaliano, Hession, Cate, Tizard, Sinclair, Foeller, Chow, Browning, Ramachandran and Pepinsky, 1986). Furthermore, lipocortin is secreted by human endometrium in organ culture (Gurpide, Markiewicz, Schatz and Hirata, 1986). The production of lipocortin by cultured human endometrium was stimulated by treatment with the glucocorticoid, dexamethasone, which increased lipocortin secretion by both proliferative and secretory endometrium, although only PGF<sub>2α</sub> production by secretory endometrium was inhibited by dexamethasone treatment (Gurpide, Markiewicz, Schatz and Hirata, 1986; Schatz, Markiewicz and Gurpide, 1986). Consequently, lipocortin may have an attenuating role in the control of PG synthesis by the guinea-pig uterus.

Touqui, Rothhut, Shaw, Fradin, Vargaftig and Russo-Marie (1986) demonstrated that in platelets, protein kinase C catalyses phosphorylation of a lipocortin-like protein, causing its inactivation, and thereby potentiates platelet thromboxane synthesis in response to appropriate stimuli (Mobley and Tai, 1985) due to the removal of the inhibitory action of lipocortin on PLA<sub>2</sub> activity. This inactivation by phosphorylation is a feature and characteristic



of lipocortins (DiRosa, Flower, Hirata, Parente and Russo-Marie, 1984). The major biochemical event that activates protein kinase C is thought to be the transient increase in diacylglycerol, generated by receptor-mediated phosphoinositide turnover (Kishimoto, Takai, Mori, Kikkawa and Nishizuku, 1980). The tumour promotor phorbol 12-myristate-13-acetate (TPA) activates protein kinase C directly, apparently by mimicking endogenous diacylglycerol (Castagna, Takai, Kaibuchi, Sano, Kikkawa and Nishizuka, 1982), and indeed protein kinase C does act as a cellular phorbol ester receptor. TPA has also been shown to stimulate PLA<sub>2</sub> activity in human neutrophils (Stocker and Richter, 1982), and in human amnion and decidual cells (Schrey and Read, 1986). Also, Okita, MacDonald and Johnston (1982) demonstrated that diacylglycerol levels increase 2-fold in human amnion with the onset of labour. The diacylglycerol may stimulate protein kinase C to phosphorylate lipocortin, so causing inactivation of lipocortin and removing its inhibitory influence on PLA<sub>2</sub> activity, and resulting in the increase in PG production measured at parturition in the amnion, decidua and myometrium (Willman and Collins, 1976).

Lipocortin has been shown to be released from human endometrium in culture. Therefore, the effects of hydrocortisone (the major glucocorticoid secreted in the guinea-pig; Fajer and Vogt, 1963) and of the phorbol ester TPA on PG output from the guinea-pig uterus need to be examined <sup>in</sup> culture.

#### Arachidonic acid release from and uptake into the precursor pool for PG synthesis

The release of arachidonic acid from some bound source is regarded as the rate-limiting step in PG synthesis by tissues. In the guinea-pig uterus, 93% of the total arachidonic acid is esterified to



phospholipids of which 80% is bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Leaver and Poyser, 1981). There is a significant decrease in the amount of arachidonic acid bound to PC, and an apparent decrease in the amount of arachidonic acid bound to PE between day 7 and day 15 of the oestrous cycle. Unesterified arachidonic acid represents only 0.1% of the total arachidonic acid content of the guinea-pig uterus (Leaver and Poyser, 1981), but even  $1\mu\text{g}$  unesterified arachidonic acid/g tissue represents a considerable pool in relation to uterine PG production. However, arachidonic acid has been reported to be rapidly released after killing and manipulation of the tissue, in guinea-pig spleen (Flower and Blackwell, 1976) and in rat cerebral cortex tissue (Marion, Pappius and Wolfe, 1979), therefore the levels of free arachidonic acid measured in the guinea-pig uterus may be even lower in vivo.

Ning, Leaver and Poyser (1983) examined the uptake of [ $^3\text{H}$ ] arachidonic acid and [ $^3\text{H}$ ]-oleic acid into guinea-pig endometrial phospholipids. Significantly more [ $^3\text{H}$ ]-arachidonic acid but not significantly more [ $^3\text{H}$ ]-oleic acid was incorporated into phospholipids (particularly PC and PE) by day 15 than by day 7 guinea-pig endometrium in culture for 24h (Ning, Leaver and Poyser, 1983). Ning and Poyser (1984) determined that PC was the major phospholipid present in the guinea-pig uterus, although more arachidonic acid was bound to PE. Lesser amounts of phosphatidylinositol (PI) and phosphatidylserine (PS) were present with sphingomyelin being present in similar quantities to PE. There were no significant changes in the amounts of any of these endometrial phospholipids between day 7 and day 15 of the oestrous cycle (Ning and Poyser, 1984). From these findings, increased incorporation of arachidonic acid into endometrial phospholipids on day 15 compared to day 7 of the cycle is not due to a

change in uterine phospholipid content. Furthermore, there is no increase in the turnover of phospholipids because the de novo synthesis of PC, the synthesis of PC by methylation of PE, and the synthesis of PI were not different between days 7 and 15 of the cycle (Ning and Poyser, 1984). Therefore, the incorporation of arachidonic acid into phospholipid seems to be by the addition to lysophospholipids rather than by de novo synthesis (see Trotter and Ferber, 1981), and it is known that arachidonic acid is incorporated into phospholipids predominantly by this mechanism (Hill and Lands, 1968; Yamashita, Hosaka and Numa, 1973). Clearly, a system exists by which the guinea-pig endometrium is able to replenish and maintain phospholipid-bound pools of arachidonic acid precursor by increasing uptake of arachidonic acid towards the end of the cycle at a time of increased  $\text{PGF}_{2\alpha}$  synthesis, to compensate for the utilization of arachidonic acid for PG synthesis, the source of which is primarily from PC and PE.

#### The action of ovarian steroid hormones on the amounts of PGH synthase present in the uterus

The amounts of  $\text{PGF}_{2\alpha}$  present in uterine tissues is very low and is inadequate to account for the large amounts of  $\text{PGF}_{2\alpha}$  synthesised and released by the uterus towards the end of the oestrous cycle in the guinea-pig (Poyser, 1972) and sheep (Wilson, Cenedella, Butcher and Inskeep, 1971). This is further evidence that prostaglandins are synthesised immediately prior to release and are not stored within the tissue.

Homogenisation of the guinea-pig uterus releases approximately  $5\mu\text{g}$  arachidonic acid/100mg tissue (Mitchell, Poyser and Wilson, 1977) and this effect is independent of the day of the oestrous cycle.

Therefore, release of substrate is not the rate-limiting step for PG synthesis in these in vitro incubates, and this was exemplified by Poyser (1972) who demonstrated that the addition of exogenous arachidonic acid did not increase the amount of PG synthesised by these uterine homogenates. PGH synthase exhibits a self catalysed destruction (Lands, LeTellier, Rome and Vanderhoek, 1971) so, as PGs are formed, the enzyme performing this conversion is inactivated. Therefore, the amount of PGs produced by homogenate preparations following incubation for 60 min, represent a direct measure of the amount of PGH synthase present in the tissue. Poyser (1973) suggested that the increase in amounts of prostaglandins formed by the uterus may reflect an increase in the amount of PGH synthase present in the uterus. Poyser (1972) demonstrated that the amounts of  $\text{PGF}_{2\alpha}$  synthesised by homogenates of guinea-pig uterus increased on day 12 of the oestrous cycle and then increased further on day 14, and these increases paralleled the increase in  $\text{PGF}_{2\alpha}$  concentrations measured in utero-ovarian venous plasma (Blatchley, Donovan, Horton and Poyser, 1972). Four to six times more  $\text{PGF}_{2\alpha}$  than  $\text{PGE}_2$  was synthesised by guinea-pig uterine homogenates. This increase in the "prostaglandin synthesizing activity" (or "synthesizing capacity", the term used in this thesis) in the guinea-pig uterus at the end of the oestrous cycle was confirmed by Wlodawer, Kindahl and Hamberg (1976), by the estimation of the amounts of [ $^{14}\text{C}$ ]-arachidonic acid converted into PGs by guinea-pig uterine microsomes.

The effects of oestrogen and/or progesterone on the  $\text{PGF}_{2\alpha}$  uterine synthesizing capacity of the ovariectomised guinea-pig was investigated by Naylor and Poyser (1975). They demonstrated that oestradiol, but not progesterone, was the stimulus to increase uterine  $\text{PGF}_{2\alpha}$  synthesizing capacity in the guinea-pig. Wlodawer, Kindahl and

Hamberg (1976), and Thaler-Dao, Ramonatxo, Saintot, Chaintreuil and Crastes de Paulet (1982) subsequently confirmed this stimulatory effect of oestradiol on the  $\text{PGF}_{2\alpha}$  synthesizing capacity of the guinea-pig uterus.

The guinea-pig uterus not only synthesises  $\text{PGF}_{2\alpha}$ , but also  $\text{PGE}_2$ ,  $\text{PGI}_2$  (measured as its stable metabolite 6-keto- $\text{PGF}_{1\alpha}$ ) and thromboxane  $\text{A}_2$  (measured as its stable metabolite  $\text{TXB}_2$ ). The synthesizing capacities of these PGs and TX of endometrium, myometrium and whole uterus were examined, from both intact animals and from ovariectomised animals treated with oestradiol and/or progesterone *in vivo* (Poyser, 1983a). In the guinea-pig endometrium, the synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{TXB}_2$  increased between day 7 and day 15 of the oestrous cycle by 2.3-fold, 2.2-fold, 1.4-fold and 1.2-fold, respectively. Between day 7 and oestrus, the synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{TXB}_2$  increased 4.4-fold, 3.0-fold, 1.6-fold and 1.3-fold, respectively. It was demonstrated that endometrial production of PGs and TX increased after day 11, with a preferential stimulation of  $\text{PGF}_{2\alpha}$  synthesizing capacity. In the myometrium, little change in the synthesizing capacities of PGs and TX were observed throughout the oestrous cycle. The major PG synthesised by homogenates of myometrium was  $\text{PGI}_2$  (measured as 6-keto- $\text{PGF}_{1\alpha}$ ), with lesser amounts of  $\text{PGF}_{2\alpha}$  and very small amounts of  $\text{PGE}_2$  and  $\text{TXB}_2$  being synthesised. The changes in production of PGs and TX by homogenates of whole guinea-pig uterus generally reflected changes in the PG synthesizing capacities of the endometrial layer of the uterus, demonstrating that the endometrium is the tissue that is controlled by steroid hormones to synthesise the uterine luteolytic hormone (Poyser, 1983a).

Progesterone treatment of ovariectomised guinea-pigs had no effect on PG or TX synthesis by homogenates of the endometrium when compared to control ovariectomised guinea-pigs (Poyser, 1983a). Oestradiol treatment stimulated the endometrial synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{TXB}_2$  by 3.2-fold, 1.6-fold, 2.2-fold and 2.0-fold, respectively (Poyser, 1983a). When progesterone and oestradiol were administered together, which is the optimum stimulus for the output of  $\text{PGF}_{2\alpha}$  from the uterus, the  $\text{PGF}_{2\alpha}$  synthesizing capacity was only stimulated 1.7-fold, and there was no stimulation of the other products (Poyser, 1983a). Progesterone treatment appeared to attenuate the stimulatory action of oestradiol in increasing endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity. Since oestradiol stimulates the synthesis of all PGs and TX by homogenates of the endometrium and since the supply of arachidonate is not rate limiting in this in vitro preparation, there has to be an increase in the concentrations of PGH synthase in the uterus to account for this general increase in PG production. Oestradiol appears to be the stimulatory factor required to increase  $\text{PGF}_{2\alpha}$  synthesizing capacity (i.e. PGH synthase concentrations) towards the end of the cycle (Poyser, 1983a). This corresponds to the increase in oestradiol output from the ovary on day 10 of the oestrous cycle (Joshi, Watson and Labhsetwar, 1973). However, the optimum stimulus for increasing  $\text{PGF}_{2\alpha}$  synthesizing capacity (i.e. only oestradiol) is not the optimum stimulus for increasing uterine  $\text{PGF}_{2\alpha}$  output (i.e. oestradiol acting on a progesterone-primed uterus), so the increase in PGH synthase levels in the endometrium after day 11 is not the main factor controlling endometrial  $\text{PGF}_{2\alpha}$  synthesis and output. Changes in PGH synthase concentration in the endometrium appear to be only secondary effects in the selective stimulation of  $\text{PGF}_{2\alpha}$  synthesis.

Treatment of the guinea-pig uterus on day 10 of the oestrous cycle in vivo with actinomycin D, a DNA-dependent RNA synthesis inhibitor, decreased the amount of  $\text{PGF}_{2\alpha}$  synthesised by uterine homogenates on day 15 of the oestrous cycle (Poyser, 1979). Addition of arachidonic acid to the uterine homogenates did not overcome this decrease in  $\text{PGF}_{2\alpha}$  synthesizing capacity, demonstrating that lack of substrate was not rate-limiting. Treatment with this protein synthesis inhibitor apparently prevented oestradiol causing the increase in PGH synthase levels in the uterus towards the end of the cycle. The treatment of guinea-pigs which had received intra-uterine actinomycin D on day 10, with exogenous oestradiol on days 11-14 did not overcome this prevention of the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity on day 15 of the oestrous cycle. As plasma progesterone concentrations remain high after actinomycin D treatment, presumably due to luteal regression being prevented by the inhibition of  $\text{PGF}_{2\alpha}$  synthesis by and release from the uterus, protein synthesis (including the synthesis of PGH synthase) appears to be a major controlling factor of uterine  $\text{PGF}_{2\alpha}$  production. However, the  $\text{PGF}_{2\alpha}$  synthesizing capacity of the guinea-pig endometrium only increases 2.5-fold between day 7 and day 15 (Poyser, 1983a) whereas the output of  $\text{PGF}_{2\alpha}$  from the guinea-pig uterus increases 22-fold over this period (Poyser and Brydon, 1983). This also demonstrates that the  $\text{PGF}_{2\alpha}$  synthesizing capacity of the guinea-pig endometrium is not the primary factor controlling endometrial  $\text{PGF}_{2\alpha}$  production, but is only of secondary importance. Early in the cycle, the potential for high  $\text{PGF}_{2\alpha}$  synthesis exists within the uterus, if uterine PGH synthase concentrations were the only controlling factor, but this potential is not expressed.

Clearly other controlling factors are involved in  $\text{PGF}_{2\alpha}$  production by the guinea-pig uterus. Studies need to be performed to investigate further the effect of protein synthesis inhibitors and ovarian steroid hormones on uterine PG production in the guinea-pig in order to elucidate some of the other intracellular factors involved in the control of endometrial  $\text{PGF}_{2\alpha}$  synthesis.

### Phospholipase Enzymes

Cellular phospholipases exist in almost every type of cell and their functions are as varied as their cellular and tissue localisation and properties (Waite, 1985). The phospholipases catalyse the hydrolysis of phospholipids, which may be considered as derivatives of glycerophosphate in which the two hydroxyls are esterified to long chain fatty acids and the phosphoryl group forms a phosphodiester bond with a polar moiety. A "stereospecific nomenclature" (sn) system is used which is based on the glycerol backbone and which is numbered as shown in Figure 1.b.

All four ester bonds in a phospholipid are susceptible to enzymatic hydrolysis. Phospholipase  $A_1$  cleaves the acyl ester at the sn-1 position. Phospholipase  $A_2$  cleaves at the sn-2 position. Phospholipase C cleaves the phosphodiester bond on the glycerol side, and phospholipase D cleaves the phosphodiester bond on the polar groups side (Dennis, 1983; see Figure 1.b).

Phospholipase  $A_1$ : Phospholipase  $A_1$  has been discovered in a wide variety of tissues. The phospholipases  $A_1$  that have been studied most are generally intracellular, and their specificity does not appear to be absolute. Their distribution and properties have been extensively reviewed by Van den Bosch (1980). However, since

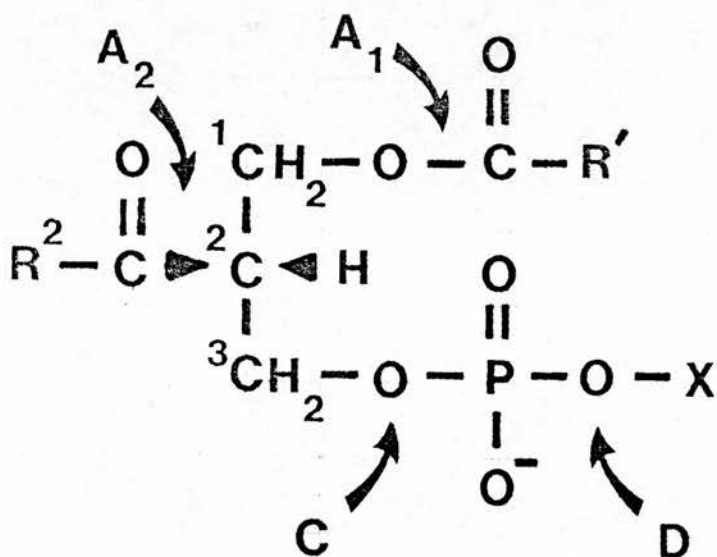


Figure 1.b

Sites of action of phospholipase A<sub>1</sub>, A<sub>2</sub>, C and D. R<sup>1</sup> and R<sup>2</sup> refer to the fatty acids on the sn-1 and sn-2 positions of the stereospecifically numbered phospholipid and X refers to the polar group - the most common are choline, ethanolamine, serine, inositol and glycerol.



Phospholipase A<sub>1</sub> only releases fatty acids from the sn-1 position, its activity is not important for the release of arachidonic acid, which is usually found esterified to the sn-2 position of a phospholipid.

Phospholipase A<sub>2</sub>: Phospholipase A<sub>2</sub> was the first phospholipase to be recognised. It was discovered by the observation that pancreatic juice or cobra venom would hydrolyse phosphatidylcholine (Wittcoff, 1951). The enzyme has subsequently been found to occur in a large variety of snake venoms, and in mammalian exocrine glands where it is involved in digestion. These extracellular phospholipases A<sub>2</sub> are much more active than intracellular phospholipase A<sub>2</sub> and their properties have been reviewed extensively by Verheij, Slotboom and de Haas (1981). Phospholipase A<sub>2</sub> exist in different forms within cells depending upon the enzymes' properties which include calcium dependence, pH optima, localisation in subcellular compartments and substrate specificity.

Phospholipase A<sub>2</sub> hydrolyses the sn-2 fatty acid from the glycerol backbone, leaving a 1-acyl lysophosphatide. Unsaturated fatty acids are found predominantly in the 2-position of phospholipids, so phospholipase A<sub>2</sub> is the important enzyme for the release of arachidonic acid from phospholipids. Rittenhouse-Simmons and Deykin (1981) demonstrated that the enzyme shows no preference for phosphatidylethanolamine over phosphatidylcholine. Conditions for optimal phospholipase A<sub>2</sub> activity vary among tissues and they are also dependent on the types of phospholipase A<sub>2</sub> present. For instance, in human platelet homogenates and membranes, these enzymes' maximum hydrolytic ability to release arachidonic acid occurred at pH 9.5 with 10mM calcium ions (Derksen and Cohen, 1975; Jesse and Cohen, 1976).

Perhaps the most detailed study which has examined phospholipase enzymes in a single cell type has been carried out in a murine macrophage cell line. Ross, Deems, Jesaitis, Dennis and Ulevitch (1985) characterised a number of different phospholipase A<sub>2</sub> activities in the murine macrophage cell line P388D<sub>1</sub>. A single Ca<sup>2+</sup>-dependent activity with a pH optimum of 8.8 was identified in membrane-rich fractions, and could be clearly separated from the remaining activities which were Ca<sup>2+</sup>-independent and exhibited pH optima of 7.5, 5.1 and 4.2. Subsequently, Ulevitch, Watanabe, Sano, Lister, Deems and Dennis (1988) purified this Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> and determined its specific activity as 1.7  $\mu\text{M min}^{-1}\text{mg}^{-1}$  which is in the range of the activities of other intracellular phospholipases A<sub>2</sub> that have been previously investigated and which range between 0.2 and 8  $\mu\text{M min}^{-1}\text{mg}^{-1}$ . In contrast, pure extracellular phospholipases A<sub>2</sub> from mammalian pancreas and snake venom possess specific activities in the range of 100  $\mu\text{M min}^{-1}\text{mg}^{-1}$ .

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity in the uterus: Downing and Poyser (1983) examined PLA<sub>2</sub> activities on day 7 and day 16 of the oestrous cycle in the guinea-pig. They showed that PLA<sub>2</sub> activity was present in both microsomal and supernatant fractions of homogenates of guinea-pig endometrium, after centrifugation at 100,000 g for 60 min, although 90% of the PLA<sub>2</sub> activity was present in the microsomal fractions and so was presumably membrane-bound. Both of these enzyme fractions had optimum enzyme activity at pH 8, but the microsomal fraction demonstrated a requirement for 7mM Ca<sup>2+</sup> for maximal enzyme activity, whereas the supernatant fraction only demonstrated a requirement for 2mM Ca<sup>2+</sup> for maximal activity. The activities of PLA<sub>2</sub> in the microsomal fractions on day 7 and day 16 of the oestrous

cycle were  $66.2 \pm 7.5$  and  $96.0 \pm 10.7$  pmol/mg protein/min, respectively. The activities of PLA<sub>2</sub> in the supernatant were  $4.2 \pm 0.8$  and  $7.8 \pm 0.9$  pmol/mg protein/min on the respective days. Therefore the activity of PLA<sub>2</sub> in the endometrium increases  $1.4 \pm 1.8$ -fold between the middle and the end of the oestrous cycle in the guinea-pig. Downing and Poyser (1983) also estimated the maximal theoretical release rates of arachidonic acid by microsomal PLA<sub>2</sub>, from the total amount of protein present in the endometrium and from the percentage of the total unsaturated fatty acids in the uterus as arachidonic acid. On days 7 and 15/16, the maximum theoretical rates of release of arachidonic acid are 15ng/min/uterine horn and 22ng/min/uterine horn, respectively. Poyser and Brydon (1983) showed that the amounts of PGF<sub>2</sub> $\alpha$  released from the guinea-pig uterus superfused in vitro on day 7 and day 15 are 0.11 ng/min/uterine horn and 2.5ng/min/uterine horn, respectively. Poyser and Brydon (1983) concluded that the rates of release of PGF<sub>2</sub> $\alpha$  from the superfused uterine horns were very similar to the outputs of PGF<sub>2</sub> $\alpha$  from the guinea-pig uterus in vivo (Poyser and Maule Walker, 1979). The endometrium clearly has a maximum potential for the release of arachidonic acid for PG production that far exceeds the observed uterine PG output, so the activity of PLA<sub>2</sub> must be controlled to regulate PG output. Furthermore, Poyser and Brydon (1983) demonstrated a 22-fold increase in uterine PGF<sub>2</sub> $\alpha$  output between days 7 and 15 of the cycle, but Downing and Poyser (1983) showed overall PLA<sub>2</sub> activity increased only 1.5-fold over this same period. Total PLA<sub>2</sub> activity cannot therefore be the controlling factor in uterine PGF<sub>2</sub> $\alpha$  production by the guinea-pig.

Phospholipase enzymes, notably PLA<sub>2</sub>, are regulated by steroid hormones. Uterine PLA<sub>2</sub> activity increases 14-fold in hypophysectomised rats receiving oestradiol-17 $\beta$  implants (Dey,

Hoversland and Johnson, 1982) and Pakrasi, Cheng and Dey (1983) demonstrated a stimulation of PLA<sub>2</sub> activity by oestradiol-17 $\beta$  implants in ovariectomised rats. In the guinea-pig, the increase in endometrial PLA<sub>2</sub> activity between days 7 and 16 of the oestrous cycle (Downing and Poyser, 1983) occurred after the rise in plasma oestradiol concentrations on day 10 (Joshi, Watson and Labhsetwar, 1973) which suggests that oestradiol is responsible for this increase. In human endometrium, Bonney (1985) demonstrated that changes in the activity of Ca<sup>2+</sup>-dependent PLA<sub>2</sub> were dependent on the stage of the menstrual cycle. The activity of PLA<sub>2</sub> was low in the early proliferative phase and increased towards mid-cycle to reach a maximum activity in the early secretory phase. It was proposed that the preovulatory surge of oestradiol, possibly with progesterone from the newly formed corpus luteum may be the stimulus for the dramatic rise in PLA<sub>2</sub> activity in the human endometrium. Subsequently PLA<sub>2</sub> activity declined in the presence of progesterone to a low level at menstruation (Bonney, 1985). A further study by Bonney, Qizalbash and Franks (1987) on the effects of steroids on human PLA<sub>2</sub> activity in explant endometrium has shown that the calcium dependent form of PLA<sub>2</sub> is stimulated by pretreatment with progesterone followed by oestradiol, which is consistent with the progesterone-priming effect. Dey, Hoversland and Johnson (1982) have also demonstrated in the rat that oestradiol administered after progesterone-priming stimulates uterine PLA<sub>2</sub> activity.

As discussed earlier, the release of arachidonic acid by PLA<sub>2</sub> is regarded as the rate limiting step in uterine PG synthesis and, without any other influencing factors, the highest PLA<sub>2</sub> activity should determine the availability of the arachidonic acid for PG production. However, in studies by Bonney (1985), the highest

activity of PLA<sub>2</sub>, which is present during the early secretory phase of the menstrual cycle, occurs before the highest production of PGs by the endometrium at the end of the cycle (Downie, Poyser and Wunderlich, 1974; Singh, Baccarini and Zuspan, 1975). Obviously, as in the guinea-pig, absolute PLA<sub>2</sub> activity in the endometrium is not the sole factor controlling arachidonic acid release, so other factors mediating uterine PLA<sub>2</sub> activity must be involved.

Calcium has been shown to be required for maximal PLA<sub>2</sub> activity in the endometrium in vitro (Downing and Poyser, 1983; Bonney, 1985), and has also been discussed as a regulator of PLA<sub>2</sub> activity in other cells (Van den Bosche, 1980). Later, the effect of increasing intracellular calcium concentrations on PG production by the guinea-pig uterus is examined.

Lysophospholipase (phospholipase B): The phospholipase B designation was used for an enzyme that was thought to cleave at both sn-1 and sn-2 positions and would hydrolyse a phospholipid as well as its lysophospholipid product, or did not have clear specificity. However, these enzymes have been difficult to characterise. There appears to be little distinction between lysophospholipases and phospholipase A<sub>1</sub> in many cases (Dennis, 1983; Waite, 1985). Lysophospholipase (lysophospholipid acyl hydrolase) is another fatty acid releasing enzyme, which removes the fatty acid from the sn-1 carbon position from a lysophospholipid. Arachidonic acid is not usually found at this site so the role of lysophospholipase is not important in the control of precursor release for prostaglandin synthesis.

Phospholipase C: An alternative pathway for the generation of free arachidonic acid demonstrated in platelets is the conversion of phosphatidylinositol by phospholipase C (PLC) to 1,2-diacylglycerol

which is then converted by diacylglycerol lipase to produce free arachidonic acid (Bell, Kennerley, Stanford and Majerus, 1979). Di Renzo, Johnston, Okazaki, Okita, MacDonald and Bleasdale (1981) have reported PLC activity in human uterine decidua, and Bonney and Franks (1987) have demonstrated PLC activity in human endometrium. Chan (1983) has reviewed the investigations on human uterine tissues and found a correlation between uterine PG generation, menstrual PG release and the severity of dysmenorrhoea in individual cases. In human endometrium, the stage of the menstrual cycle has no effect on PLC activity, although an increase in PLC may be involved in some pathological conditions including menorrhagia and dysfunctional uterine bleeding, which are associated with increased concentrations of PGs in the endometrium (Bonney and Franks, 1987).

Phospholipase C metabolises phosphatidylinositol to 1,2-diacylglycerol, and so it must also be considered that other enzymes involved in this release of arachidonic acid, for instance diacylglycerol lipase, may be controlling precursor release. Leaver and Poyser (1981) demonstrated that phosphatidylinositol contains less than 10% of the total arachidonic acid in the guinea-pig uterus, and Ning and Poyser (1984) demonstrated there was no change in the turnover of phosphatidylinositol between day 7 and day 15 of the oestrous cycle. Therefore, it appears that the action of phospholipase C on phosphatidylinositol is not involved in the release of arachidonic acid for PG synthesis by the guinea-pig uterus. Furthermore, Poyser (1987a) examined the effects of PLA<sub>2</sub> and PLC on PG synthesis and release by the guinea-pig uterus superfused in vitro, and demonstrated both PLA<sub>2</sub> and PLC stimulated PG output from uteri removed on day 7 and day 15 of the oestrous cycle. However, the PLC used was bacterial in origin which preferentially used

phosphatidylcholine, instead of phosphatidylinositol as substrate. A phosphatidylcholine-specific PLC is present in some mammalian tissues (Clark, Shorr and Bromalaski, 1986). There were differences in the amounts and proportions of the different PGs released by PLA<sub>2</sub> and PLC treatment of guinea-pig uteri. PLA<sub>2</sub> appeared to produce a similar pattern of PG release to the pattern produced by the action of calcium using the calcium ionophore, A23187, whereas PLC treatment produced a greater overall stimulation of PG synthesis and, specifically, a much greater stimulation of PGE<sub>2</sub> production. Poyser (1987a) concluded that the stimulation of endogenous PLA<sub>2</sub> by Ca<sup>2+</sup>, rather than the stimulation of endogenous PLC, is involved in the release of arachidonic acid for PG synthesis by the guinea-pig uterus. However, care must be taken in the interpretation of these data due to the different properties of intracellular PLA<sub>2</sub> and the extracellular PLA<sub>2</sub> used in this study, and also to the differences in substrate specificity of the PLC used.

#### The role of calcium in the stimulation of uterine PG synthesis

Membrane bound PLA<sub>2</sub> shows a requirement for calcium which was demonstrated by Kunze, Bohn and Vogt (1974) in homogenates of bovine seminal vesicles. The synthesis of PGs by bovine seminal vesicles was stimulated with calcium and this stimulatory effect was inhibited by EDTA. A small amount of PG was formed even in the presence of EDTA, but this was thought to reflect the pool of free precursor present in the homogenate.

The calcium ionophore, A23187 stimulates PG production by several tissues (Knapp, Oelz, Roberts, Sweetman, Oats and Reep, 1977). Leaver and Seawright (1982) demonstrated that A23187 stimulates PGF<sub>2α</sub> output from cultured day 7 and day 15 guinea-pig endometrium in a



similar way to the addition of exogenous arachidonate precursor to the tissue. It was proposed that calcium influx into the cell regulates the availability of substrate because of the similarity of this effect with the addition of exogenous arachidonate to the culture medium. Subsequently, Poyser and Brydon (1983) demonstrated the role of calcium in the stimulation of PG synthesis by the guinea-pig uterus. The outputs of  $\text{PGF}_{2\alpha}$ , 6-keto- $\text{PGF}_{1\alpha}$  and, to a lesser extent,  $\text{PGE}_2$  were stimulated by the administration of A23187 to both day 7 and day 15 guinea-pig uteri superfused in vitro.  $\text{PGF}_{2\alpha}$  output was stimulated 4-fold and 1.9-fold on days 7 and 15, respectively. Poyser (1983a) has reported that the main sources of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  in the guinea-pig uterus are the endometrium and the myometrium, respectively, and these tissues are most likely the sources of these PGs when the uterus is being superfused and stimulated with A23187 (Poyser, 1983b).

Interestingly however, the high basal output of  $\text{PGF}_{2\alpha}$  from day 15 guinea-pig uterus superfused in vitro was not affected by using calcium free Krebs' solution (Poyser, 1984a). The output of  $\text{PGE}_2$  was also unaffected, whereas  $\text{PGI}_2$  synthesis was inhibited by superfusing with  $\text{Ca}^{2+}$ -free medium. This last result is in agreement with Vesin, Lieber and Harbon (1982) who demonstrated that  $\text{PGI}_2$  production by the guinea-pig myometrium is dependent on extracellular  $\text{Ca}^{2+}$ . However, the stimulation of the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  by A23187 was abolished using  $\text{Ca}^{2+}$ -free medium which showed that the action of A23187 is dependent on extracellular  $\text{Ca}^{2+}$ , and that A23187 is acting as a calcium ionophore and not by some other unspecified mechanism (Poyser, 1984a). In this series of experiments, the guinea-pig uteri were only superfused for 2.5 hours with  $\text{Ca}^{2+}$ -free medium, and it is possible that there was insufficient time for an



intracellular source of calcium in the endometrium to be depleted so therefore a decline in basal  $\text{PGF}_{2\alpha}$  output was not observed. Using an intracellular calcium antagonist TMB-8, Poyser (1985b) demonstrated that the A23187-induced increase in the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 15 superfused guinea-pig uterus were inhibited. However, the high basal output of  $\text{PGF}_{2\alpha}$  was unaffected by TMB-8 treatment. TMB-8 was only administered to the superfused guinea-pig uterus for 40 min, and this may be insufficient time for an inhibitory action of TMB-8 to sequester intracellular calcium and thus making it unavailable for stimulation of  $\text{PGF}_{2\alpha}$  synthesis and release. Consequently, the long-term effects of lack of  $\text{Ca}^{2+}$  and of TMB-8 on uterine PG synthesis need to be examined.

However, very high calcium concentrations are required for the direct stimulation of  $\text{PLA}_2$ , and these concentrations are not reached in the whole cell. However, it may be speculated that discrete sites ~~out~~with the cell may be able to maintain these raised  $\text{Ca}^{2+}$  levels. Recent evidence has gathered for a further mechanism of controlling  $\text{PLA}_2$  by  $\text{Ca}^{2+}$  ions, by the modulation of  $\text{PLA}_2$  by calmodulin. This is examined in the next section.

#### The role of calmodulin in mediating $\text{PLA}_2$ activity

Calcium has been shown to mediate prostaglandin output from human dispersed amnion cells (Olson, Opavsky and Challis, 1983), and calcium stimulates the activity of amnion phospholipases A<sub>2</sub> and C (Schultz, Schwartz, MacDonald and Johnston, 1975; DiRenzo, Johnston, Okazaki, Okita, MacDonald and Bleasdale, 1981) which directly or indirectly mobilise arachidonic acid for PG synthesis. However, the optimum stimulatory concentrations of  $\text{Ca}^{2+}$  are in the millimolar range,

whereas intracellular calcium concentrations are generally in the micromolar range. Therefore, it may seem unlikely that  $\text{Ca}^{2+}$  is activating  $\text{PLA}_2$  directly, but acts via a mediator.

Calmodulin is a small acidic protein (M.W. 16,700) consisting of a single polypeptide chain with four loops, each loop possessing a domain for binding a calcium ion. Calmodulin plays an important role in the activation and regulation of many  $\text{Ca}^{2+}$ -dependent processes in eukaryotic cells, including the control of several enzyme activities (Cheung, 1980). Wong and Cheung (1979) using  $\text{PLA}_2$  from human platelet membranes and Moskowitz, Andres, Silva, Shapiro, Schook and Puszkin (1985) using snake venom  $\text{PLA}_2$  demonstrated that maximal enzymatic activity of  $\text{PLA}_2$  was stimulated by calcium which was mediated via calmodulin, thus demonstrating that  $\text{PLA}_2$  is a calcium and a calmodulin dependent enzyme. Wong, Lee, Chao and Cheung (1980) showed that the addition of exogenous calmodulin stimulated  $\text{TXB}_2$  production by human platelets.

The binding of calcium to calmodulin creates a hydrophobic domain on the surface of the calmodulin, which appears necessary for the interaction of calmodulin with its receptor protein, and this hydrophobic domain is thought to be the binding site of the calmodulin inhibitor trifluoperazine (TFP; Andersson, Drakenberg, Thulin and Forsen, 1983). Furthermore, calmodulin has been implicated as a mediator of prostaglandin synthesis, because TFP prevented the release of arachidonic acid and the stimulation of PG synthesis by A23187, vasopressin, NaCl and mannitol in rat renal medulla (Craven and DuRupertis, 1983), and TFP inhibited the release of arachidonic acid from phosphatidylcholine by  $\text{PLA}_2$  after thrombin stimulation of platelets (Walenga, Opas and Feinstein, 1981). Addition of TFP to dispersed human amnion cells obtained after caesarian section

inhibited PGE<sub>2</sub> output during 3h in culture, and TFP also inhibited A23187-stimulated PGE<sub>2</sub> output from these cells. These studies indicate that calmodulin controls the activity of PG synthesis in the human amnion (Olson, Challis, Opavsky, Smeija, Kramar and Skinner, 1985). Poyser (1985a) demonstrated that A23187-stimulated output of PGF<sub>2</sub> $\alpha$  from the guinea-pig uterus superfused in vitro was inhibited by TFP treatment on days 7 and 15 of the oestrous cycle. Another calmodulin antagonist, W-7, also prevented the A23187-induced production of PGF<sub>2</sub> $\alpha$  output from the guinea-pig uterus superfused in vitro (Poyser, 1985b). These studies implicate a role for calmodulin in the control of PLA<sub>2</sub>, and hence PGF<sub>2</sub> $\alpha$  synthesis by the guinea-pig uterus. However, calmodulin inhibitors failed to inhibit basal PGF<sub>2</sub> $\alpha$  output when superfused over the guinea-pig uterus for 40 min. Consequently, the longer term effect of calmodulin inhibitors needs to be investigated to determine whether calmodulin is involved in the control of the increase in basal PGF<sub>2</sub> $\alpha$  output from the guinea-pig uterus which occurs in vivo towards the end of the cycle.

#### SUMMARY

PGF<sub>2</sub> $\alpha$  is the uterine luteolytic hormone in the guinea-pig. Oestradiol acting on a progesterone-primed uterus is the optimum stimulus for the increase in PGF<sub>2</sub> $\alpha$  output from the guinea-pig uterus towards the end of the oestrous cycle in vivo. The synthesis of PGF<sub>2</sub> $\alpha$  is primarily controlled by the activity of PLA<sub>2</sub> in releasing arachidonic acid, the substrate for PG production. Calcium and also calmodulin have been suggested as mediators of PLA<sub>2</sub> activity. Also, steroid hormones act by inducing the production of new proteins, and proteins have been implicated in the control of PG synthesis.



Consequently, in this thesis, the roles of the following factors in the control of  $\text{PGF}_{2\alpha}$  (and also  $\text{PGE}_2$  and  $\text{PGI}_2$ ) synthesis by and release from the guinea-pig uterus have been studied.

- a. Calcium and calmodulin.
- b. Protein synthesis.
- c. Steroid hormones and oxytocin.

The general techniques used in these experiments presented in this thesis are used routinely and are described in detail in the next section. Specific experimental methods are then described in the subsequent results chapter.

## SECTION 2

### MATERIALS AND GENERAL METHODS

#### 2.1 INTRODUCTION

The work presented in this thesis has involved the measurement of PG output from the guinea-pig uterus superfused in vitro, PG output from guinea-pig endometrial and myometrial tissue maintained in tissue culture, and PG production by homogenates of guinea-pig endometrial and myometrial tissue following incubation in vitro. Also the incorporation of [ $^3\text{H}$ ]-leucine into cultured endometrial tissue has been assessed as a measure of protein synthesis.

PGs were extracted from perfusates and incubates of homogenates by organic solvents, and were measured by radioimmunoassay (RIA). PGs released into tissue culture medium were measured directly, without extraction, by RIA. The experimental work has been divided into six sections. In this chapter, the general procedures for superfusing whole uterine horns in vitro, for homogenising and incubating uterine tissue, for measuring the incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted proteins by cultured uterine tissues, and for extracting and measuring PGs and progesterone by RIA have been described.

##### 2.1.1 Materials

###### i. Solvents

Absolute ethanol - J. Borrough Ltd., London, UK

Acetone - Charles Tennent and Co. Ltd., Glasgow, UK

2-Ethoxyethanol (reagent grade) - Fisons p.l.c., Loughborough,  
UK

\* Ethyl acetate (reagent grade) - Rathburn Chemicals Ltd.,  
Walkerburn, UK

Methanol (Analar) - Rathburn Chemicals Ltd.,

Walkerburn, UK

\* Petroleum spirit, b.pt. - 40-60°C (Analar) - BDH Chemicals Ltd., Poole, UK

Toluene (reagent grade) - May and Baker Ltd., Dagenham, UK

\* redistilled before use.

ii. Radioactive Compounds

L-[4,5-<sup>3</sup>H]leucine (specific activity 120 Ci/mmol)

[5,6,8,11,12,14,15(n)-<sup>3</sup>H]prostaglandin F<sub>2α</sub> (specific activity 180 Ci/mmol)

[5,6,8,11,12,14,15(n)-<sup>3</sup>H]prostaglandin E<sub>2</sub> (specific activity 185 Ci/mmol)

6-keto[5,8,9,11,12,14,15(n)-<sup>3</sup>H]prostaglandin F<sub>1α</sub> (specific activity 170 Ci/mmol)

[1,2,6,7,16,17-<sup>3</sup>H]progesterone (specific activity 125 Ci/mmol)

All radioactive compounds were supplied by Amersham International Ltd., Cardiff, UK.

[<sup>3</sup>H]PG and [<sup>3</sup>H]progesterone stock solutions were stored at -20°C. [<sup>3</sup>H]-leucine stock solutions were stored at 4°C.

PG and progesterone stock solutions were diluted to 5μCi/ml and stored at -20°C before using for radioimmunoassay. [<sup>3</sup>H]PGF<sub>2α</sub> and [<sup>3</sup>H]PGE<sub>2</sub> were diluted with methanol; [<sup>3</sup>H]6-keto-PGF<sub>1α</sub> was diluted with acetonitrile : water (9 : 1 v/v), and [<sup>3</sup>H]progesterone was diluted with toluene.

iii. Other Chemicals

A23187 - Sigma Chemical Co., Poole, UK

Acetonitrile - Sigma Chemical Co., Poole, UK

Actinomycin D - Sigma Chemical Co., Poole, UK

Amphotericin B ('Fungizone') - Flow Labs., Irvine, UK

Arachis oil - BDH Chemicals Ltd., Poole, UK

Bovine serum albumin (BSA) - Sigma Chemical Co., Poole, UK  
 2-(4-tert-Butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (Butyl-PBD) - Koch-Light Labs., Ltd., Colnbrook, UK  
 Calcium chloride - BDH Chemicals Ltd., Poole, UK  
 Cycloheximide - Sigma Chemical Co., Poole, UK  
 2,5-Diphenyloxazole (PPO) - BDH Chemicals Ltd., Poole, UK  
 Ethyleneglycol-bis-( $\beta$ -aminoethyl ether)N,N'-tetra-acetic acid (EGTA) - Sigma Chemical Co., Poole, UK  
 Fluanisone ('Hypnorm') - Crown Chemicals Co. Ltd., Lamberhurst, UK  
 Gelatine - BDH Chemicals Ltd., Poole, UK  
 D-Glucose - BDH Chemicals Ltd., Poole, UK  
 Glutamine - Flow Labs., Irvine, UK  
 Heparin - Weddel Pharmaceuticals Ltd., London, UK  
 Hydrochloric acid (HCl) - Fisons p.l.c., Loughborough, UK  
 Hydrocortisone - Sigma Chemical Co., Poole, UK  
 Indomethacin - Merck, Sharp & Dohme Res. Labs., Hoddesdon, UK  
 Kanamycin - Flow Labs., Irvine, UK  
 L-Leucine - Sigma Chemical Co., Poole, UK  
 Magnesium sulphate - BDH Chemicals Ltd., Poole, UK  
 Medium 199 (modified) with Earle's salts - Flow Labs., Irvine, UK  
 Medium 199 (modified) with Earle's salts (calcium depleted) - Flow Labs., Irvine, UK  
 2-Mercaptoethanol - BDH Chemicals Ltd., Poole, UK  
 Midazolam ('Hypnovel') - Roche Products Ltd., Welwyn Garden City, UK  
 Naloxone ('Narcan') - Du Pont (UK) Ltd., Stevenage, UK  
 Nifedipine - Bayer (UK) Ltd., Newbury, UK

17 $\beta$ -Oestradiol - Sigma Chemical Co., Poole, UK  
 Oestradiol benzoate - Sigma Chemical Co., Poole, UK  
 Oxytocin - Sigma Chemical Co., Poole, UK  
 Phorbol 12-myristate 13-acetate (TPA) - Sigma Chemical Co.,  
 Poole, UK  
 Potassium chloride - Fisons p.l.c., Loughborough, UK  
 Potassium dihydrogen orthophosphate - BDH Chemicals Ltd., Poole,  
 UK  
 Progesterone - Sigma Chemical Co., Poole, UK  
 Puromycin - Sigma Chemical Co., Poole, UK  
 Sodium arachidonate - Sigma Chemical Co., Poole, UK  
 Sodium azide - BDH Chemicals Ltd., Poole, UK  
 Sodium chloride - BDH Chemicals Ltd., Poole, UK  
 Sodium chloride sterile solution (9g/l) - Boots Co. Ltd.,  
 Nottingham, UK  
 Sodium dodecyl sulphate (SDS) - Sigma Chemical Co., Poole, UK  
 Sodium hydrogen phosphate - BDH Chemicals Ltd., Poole, UK  
 Sodium hydrogen phosphate - BDH Chemicals Ltd., Poole, UK  
 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate  
 hydrochloride (TMB-8) - Aldrich Chemical Co. Ltd., Gillingham,  
 UK  
 Trichloroacetic acid - BDH Chemicals Ltd., Poole, UK  
 Trifluoperazine (TFP) - Smith Kline & French Labs., Welwyn  
 Garden City, UK  
 Tris(hydroxymethyl)-methylamine - BDH Chemicals Ltd., Poole, UK  
 Urea - BDH Chemicals Ltd., Poole, UK  
 Verapamil - Knoll AG, Ludwigshafen, F.R.G.  
 N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) -  
 Beecham-Wulfring, Gronau (Leine), F.R.G.



Authentic PGs and their metabolites were supplied by Dr. J. Pike, Upjohn Co., Kalamazoo, U.S.A.

iv. Gases

95% air and 5% CO<sub>2</sub>

95% O<sub>2</sub> and 5% CO<sub>2</sub>

All gases supplied by British Oxygen Co. Ltd., Guildford, U.K.

v. Other materials

Petri dishes (vented, 5cm diameter) Sterilin Ltd., Teddington, UK

Scintillation vials (3ml) Sarstedt Ltd., Leicester, UK

Micropipette tips L.I.P. Ltd., Shipley, UK and Gilson, Villiers-le-Bel, France

Donkey anti-rabbit serum (DARS) and normal rabbit serum (NRS) were obtained from the Scottish Antibody Production Unit, Carstairs Hospital, UK. Stock solutions of antisera were stored at -20°C. Stock solutions were diluted with the appropriate diluent and stored at 4°C.

2.1.2 Composition of Solutions

i. Krebs' solution (in 5 litres).

10.5g D-Glucose

10.5g NaHCO<sub>3</sub>

34.5g NaCl

8ml 10% w/v KH<sub>2</sub>PO<sub>4</sub> solution

17.7ml 10% w/v KCl solution

14.5ml 10% w/v MgSO<sub>4</sub> .7H<sub>2</sub>O solution

12.6ml 1M CaCl<sub>2</sub>

Krebs' solution was stored at 4°C

ii. Solution for PGF<sub>2α</sub> radioimmunoassay.

0.05M Tris buffer pH 8

0.1g/l Sodium azide

1.0g/l Gelatine

This solution is subsequently called "Tris diluent pH 8"

- iii. Solution for PGE<sub>2</sub> and progesterone radioimmunoassay.

0.05M Phosphate (6.9g Na<sub>2</sub>HPO<sub>4</sub> (anhydrous) + 1.7g NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O/l)  
buffer pH 7.5

0.1g/l Sodium azide

1.0g/l Gelatine

This solution is subsequently called "phosphate diluent pH 7.5".

- iv. Solution for 6-keto-PGF<sub>1α</sub> radioimmunoassay.

0.05M Tris buffer pH 6.8

0.1g/l Sodium azide

1.0g/l Gelatine

This solution is subsequently called "Tris diluent pH 6.8".

All diluents were stored at 4°C.

- v. Scintillation fluid for PG radioimmunoassays and for measurement of [<sup>3</sup>H]-leucine incorporation into proteins.

1.5l Toluene

0.9l 2-Ethoxyethanol

10.5g 2,5-Diphenoxylloxazole (PPO)

- vi. Scintillation fluid for progesterone radioimmunoassay

2.5l Toluene

10g Butyl-PBD

## 2.2 METHODS

### 2.2.1 Animals used

All animals used in these studies were virgin female guinea-pigs weighing between 600-1000g, and they were housed in a 14h light and 10h dark cycle (lights on between 0500-1900). They were offered a

standard diet without restriction of RPG pellets (Labsure, Manea, UK), hay, supplemented vegetables and water supplemented with ascorbic acid. The guinea-pigs were examined daily and a vaginal smear was taken when the vaginal membrane was open. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification is at a maximum (Nicol and Snell, 1954). All animals had exhibited at least two normal cycles before being used.

Each guinea-pig was killed by stunning and incising the neck. A blood sample was taken from the incised neck when appropriate, in order to measure the plasma progesterone concentration by radioimmunoassay (section 2.3.5). Animals on either day 7 or day 15 of the oestrous cycle were used, these being days of low and high  $\text{PGF}_{2\alpha}$  output from the uterus respectively (Blatchley, Donovan, Horton and Poyser, 1972). The uterus was rapidly removed and placed either in sterile culture medium prior to dissection for tissue culture, or in cold ( $4^{\circ}\text{C}$ ) Krebs' solution prior to superfusion or homogenisation.

#### 2.2.2 Extraction of PGs from perfusates and homogenates

PGs were extracted from superfusates and homogenates using the method described by Poyser (1972). The pH of the perfusate or homogenate medium was lowered to between pH 3.5 and 4.0 by the dropwise addition of 1M HCl. The medium was extracted twice with two volumes of ethyl acetate. These ethyl acetate fractions were combined and evaporated to dryness under reduced pressure at  $45^{\circ}\text{C}$  on a rotary evaporator. Superfusate and homogenate residues were redissolved in 10ml and 5ml ethyl acetate, respectively, and were stored at  $-20^{\circ}\text{C}$  before the amounts of PGs present in the samples were measured by radioimmunoassay (section 2.3).

This extraction procedure gives a high rate of recovery for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ . The recoveries (mean  $\pm$  s.e.m.,  $n = 3$ ) of the appropriate radioactive PGs at pH 4 were found to be  $94 \pm 3\%$  for  $\text{PGF}_{2\alpha}$ ,  $92 \pm 5\%$  for  $\text{PGE}_2$  (Poyser and Scott, 1980) and  $82 \pm 2\%$  for 6-keto- $\text{PGF}_{1\alpha}$  (Swan and Poyser, 1983). PG concentrations measured in perfusates and homogenates have not been corrected for these losses during recovery.

#### 2.2.3 Superfusion of the guinea-pig uterus in vitro

Superfusion of the guinea-pig uterus was first described by Poyser and Brydon (1983). By this technique, basal PG outputs can be measured from a single uterine horn, and also compounds can be added to the perfusing solution to examine their effects on PG production. Furthermore, each uterine horn from the same animal can be superfused simultaneously under the same conditions, with one horn remaining untreated and acting as a control for the treated horn.

After removal, each horn was weighed after blotting off surface Krebs' solution, "opened" by cutting longitudinally, and suspended in an organ chamber attached to an isotonic lever exerting a load of 2g (see Fig.2.2.3.a). The uterine horn was superfused (5ml/min) with Krebs' solution at  $37^\circ\text{C}$ , pregassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . Samples of superfusate were collected every 10 min during the specified sampling period. PGs were extracted from the superfusate with ethyl acetate (see section 2.2.2) and were measured by radioimmunoassay (see section 2.3).

#### 2.2.4 Measurement of the synthesizing capacities of PGs by homogenates of uterine tissue

This technique of homogenisation of the whole uterus of the

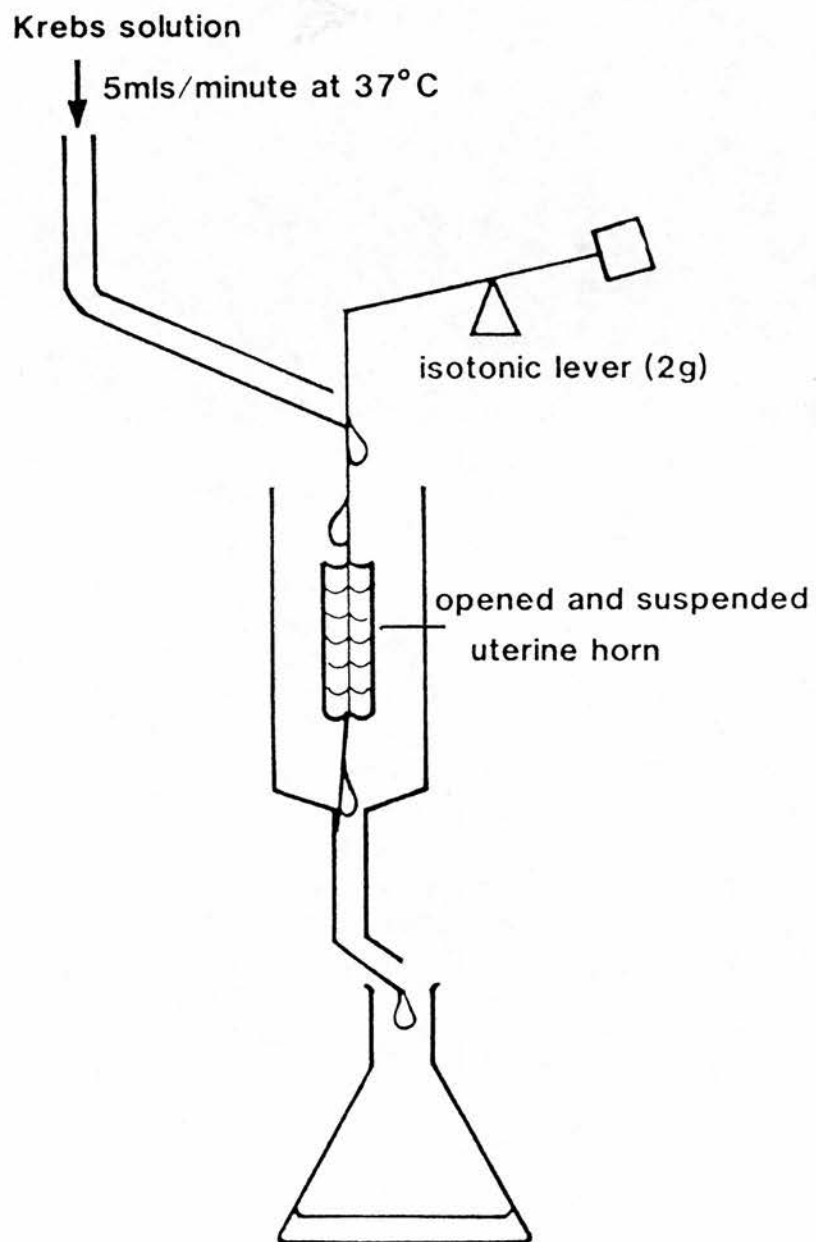


Figure 2.2.3.a

Method for the superfusion of a uterine horn in vitro.

guinea-pig to measure the capacity of the tissue to synthesize PGs was first performed by Poyser (1972). This technique was further refined by dissecting the endometrium from the myometrium in the guinea-pig uterus and measuring their synthesizing capacities separately (Poyser, 1983a). The method of homogenising endometrial and myometrial tissue either immediately after removal from the animal or after superfusion of the uterus, and of homogenising endometrium after tissue culture are described here.

i. Homogenisation of endometrium and myometrium to measure PG synthesizing capacity

The endometrium was carefully dissected away from the myometrium with a fine pair of scissors. Tissue separation by this technique is greater than 85% (Leaver and Poyser, 1981). Each tissue type was weighed after blotting dry, cut finely, and homogenised in 5ml Krebs' solution using a Fisons or Jencons glass homogeniser (for composition of Krebs' solution see Section 2.1.2). The homogenate was decanted into a 50ml conical flask. The homogeniser was then washed with a further 5ml Krebs' solution, the washings being again homogenised and then added to the flask (total volume in flask = 10ml). The flask top was covered with Nescofilm (which was perforated twice; one hole for the gassing tube and one hole to release the gas) and the flask was incubated for 60 min at 37°C in a Grant water bath, under agitation and whilst 95% O<sub>2</sub>/5% CO<sub>2</sub> was being bubbled through the Krebs' solution. After the incubation period, PGs were extracted from the incubates with ethyl acetate (section 2.2.2) and were measured by RIA (section 2.3).

ii. Homogenisation of small amounts of endometrium to measure its PG synthesizing capacity after tissue culture

The endometrium was blotted dry, weighed and homogenised by the

same procedure as above (2.2.4.i.) except that two volumes of 2.5ml Krebs' solution were used for each homogenisation, and the homogenates were poured into and incubated in a 25ml conical flask. Sodium arachidonate (2 $\mu$ g in 10 $\mu$ l ethanol) was added to each flask. The homogenates were incubated for 60 min at 37°C whilst being gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. After the incubation period PGs were extracted from the incubates with ethyl acetate (section 2.2.2) and were measured by RIA (section 2.3).

#### 2.2.5 Tissue Culture Technique

Tissue was cultured by a method based on that described by Baker and Neal (1969) for ovarian tissue, and further refined for the culture of endometrial tissue by Abel and Baird (1980), Leaver and Seawright (1982) and Ning, Leaver and Poyser (1983).

##### i. Tissue culture medium

The medium used in this technique is Medium 199, which has been modified by including Earle's salts (Morgan, Morton and Parker, 1950). Earle's balanced salt solution (EBSS) consists of a synthetic mixture of inorganic salts supplemented with glucose. The functions of this balanced salt solution are threefold:

- (a) as a buffer to maintain pH. Sodium bicarbonate (NaHCO<sub>3</sub>) (2.2g/l) is present as this buffer.
- (b) to maintain osmotic pressure.
- (c) as an energy source, provided here by glucose.

The EBSS receives further supplements of amino acids, vitamins, salts, minerals and extra organic supplements to form Medium 199 (modified); its constituents are shown in table 2.2.5.i. Glutamine is an essential factor for stromal viability and good epithelial preservation (see Kaufman, Adamec, Walton, Carney, Melin, Genta, Mass,

Ingredient	199 with Earle's Salts mg/litre	Ingredient	199 with Earle's Salts mg/litre
L-Alanine	25.00	p-Aminobenzoic acid	0.05
L-Aragine HCl	70.00	Pyridoxal HCl	0.025
L-Aspartic acid	30.00	Pyridoxine HCl	0.025
L-Cysteine HCl	0.0987	Riboflavin	0.01
L-Cysteine disodium salt	23.66	Thiamin HCl	0.01
L-Glutamic acid	66.82	DL- $\alpha$ -tocopherol phosphate disodium salt	0.01
L-Glutamine	100.0	Vitamin A acetate	0.1147
Glutathione	0.05	CaCl <sub>2</sub> 2H <sub>2</sub> O	264.9
Glycine	50.00	Fe(NO <sub>3</sub> ) <sub>3</sub> 9H <sub>2</sub> O	0.10
L-Histidine HCl H <sub>2</sub> O	21.88	KCl	400.0
L-Hydroxyproline	10.00	KH <sub>2</sub> PO <sub>4</sub>	
L-Isoleucine	20.00	MgSO <sub>4</sub> 7H <sub>2</sub> O	200.0
L-Leucine	60.00	NaCl	6800
L-Lysine HCl	70.00	NaHCO <sub>3</sub>	2200
L-Methionine	15.00	NaH <sub>2</sub> PO <sub>4</sub> 2H <sub>2</sub> O	158.3
L-Phenylalanine	25.00	Na <sub>2</sub> HPO <sub>4</sub>	
L-Proline	40.00	Adenine sulphate	10.00
L-Serine	25.00	5-AMF	0.20
L-Threonine	30.00	ATP disodium salt	10.00
L-Tryptophan	10.00	Cholesterol	0.20
L-Tyrosine - Boc derivative	29.72	2-Deoxyribose	0.50
L-Valine	25.00	D-Glucose	1000
L-Ascorbic acid	0.05	Guanine HCl	0.30
Biotin	0.01	Hypoxanthine	0.30
Celestrol	0.10	D-Ribose	0.50
D-Calcium pantothenate	0.01	Sodium acetate	36.71
Choline chloride	0.70	Phenol red sodium salt	12.00
Folic acid	0.01	Thymine	0.30
Inositol	0.05	Tween 80	5.00
Monophosphoric sodium bisulphate 3H <sub>2</sub> O	0.019	Uracil	0.30
Nicotinic acid	0.025	Xanthine	0.30
Nicotinamide	0.025		

**Table 2.2.5.i**

The constituents of Medium 199.



Dorman, Rodgers, Photopulos, Powell and Grisham, 1980), but it is only stable below 0°C. The culture medium was stored at 4°C and glutamine was added when required. Furthermore, the medium may be susceptible to bacterial and fungal growth, so antibiotics are added (Abel and Baird, 1980; Leaver and Seawright, 1982). Consequently, to 0.5l Medium 199 (modified) under sterile conditions, the following supplements were added:

glutamine (200mM; 4ml)

amphotericin B ('Fungizone') (250µg/ml; 3ml)

kanamycin (5000µg/ml; 3ml)

After mixing, the medium was dispersed into sterile 25ml storage bottles and stored at -20°C until required.

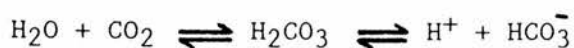
#### ii. Preparation of tissue

Endometrium was dissected away from the myometrium using fine scissors. Small pieces (1-2mm<sup>3</sup>) of endometrial or myometrial tissue were placed on a sterile lens paper lying across a sterile stainless steel grid in a sterile vented Petri dish (see Figure 2.2.5.a). The dish contained 4ml supplemented Medium 199 (modified) together with any other compounds according to the nature of the experiment, as detailed later in the results section. This arrangement allows the tissue to be held above the medium but allows medium to be readily available to the tissue by capillary action through the lens paper. Between 10 and 25mg endometrial or myometrial tissue were used in experiments measuring PG output into the medium, and between 30 and 60mg endometrial tissue were used in experiments measuring protein synthesis by [<sup>3</sup>H]-leucine incorporation.

The appropriate number of Petri dishes according to the experiment were inserted into racks, and the racks were then placed in modified Kilner jars (see figure 2.2.5.a). The air in the jars was

replaced by a 1:1 mixture of air/CO<sub>2</sub> (95% : 5%) and O<sub>2</sub>/CO<sub>2</sub> (95% : 5%) at a pressure of 0.7kgcm<sup>-2</sup> (10 lb in<sup>-2</sup>). The jars were then incubated at 37°C for the specified time. When the medium in the dishes was removed and replaced with fresh medium, the jars were regassed and then replaced in the incubator. Fresh medium was prewarmed to 37°C before being used for replacing the original medium.

Organ culture methods require the tissue to be placed at the gas/liquid interface to maximize gas exchange (Trowell, 1959). High oxygen levels are used to overcome problems of diffusion related to the size of the tissue pieces. The small amount of CO<sub>2</sub> is necessary to maintain pH. Atmospheric CO<sub>2</sub> will regulate dissolved CO<sub>2</sub> directly according to the temperature, and the CO<sub>2</sub> combines with H<sub>2</sub>O to produce H<sub>2</sub>CO<sub>3</sub> which dissociates:



HCO<sub>3</sub><sup>-</sup> has a fairly low dissociation constant with the available cations, so it tends to reassociate leaving the medium acid. The net result of increasing CO<sub>2</sub> is to depress the pH and by increasing the bicarbonate concentration the equation is pushed to the left until an equilibrium is reached at pH 7.4. Without CO<sub>2</sub> present, at this incubation temperature, the pH increases, the sodium phenol red indicator changes from red to purple and cell functions may be inhibited. In the experiments performed in this thesis, the indicator always maintained its red colour.

### iii. Tissue viability

Previous studies performed in this laboratory have carried out extensive histological studies to compare visually the viability of cultured and non-cultured guinea-pig endometrium. There were no obvious differences between tissue before and after 72h of culture (Leaver and Seawright, 1982; Ning, Leaver and Poyser, 1983; Ning and

Poyser, 1984). Therefore, this tissue culture procedure is adequate for normal maintenance and survival of the endometrium for up to 72h of culture, the longest period used in the studies to be described in this thesis.

## 2.3 RADIOIMMUNOASSAY METHODS

### 2.3.1 Measurement of PGs by radioimmunoassay (general method)

#### Introduction

The procedure for preparing radioimmunoassays to measure  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  is essentially the same. This general method will be described, with the specific details of each PG assay being noted.

#### Antisera used and assay protocol

Antisera for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were all raised in rabbits using, respectively, a  $\text{PGF}_{2\alpha}$ -bovine serum albumin conjugate (Dighe, Emslie, Henderson, Rutherford and Simon, 1975), a  $\text{PGE}_2$ -thyroglobulin conjugate (Dighe, Smith, Ungar and Whelpdale, 1978) and a 6-keto- $\text{PGF}_{1\alpha}$ -thyroglobulin conjugate (Dighe, Jones and Poyser, 1978).

The amount of [ $^3\text{H}$ ]PG used in each assay gave between 15,000-20,000 counts per tube when scintillation counted for 4 min ([ $^3\text{H}$ ]  $\text{PGF}_{2\alpha}$  and [ $^3\text{H}$ ] 6-keto- $\text{PGF}_{1\alpha}$ ) or for 10 min ([ $^3\text{H}$ ]  $\text{PGE}_2$ ). The [ $^3\text{H}$ ]PG solution was prepared by evaporating off the solvent carrier in a stream of air and then redissolving the [ $^3\text{H}$ ]PG in sufficient of the appropriate diluent, such that a 50 $\mu\text{l}$  aliquot of this solution provided the required number of counts.

The antiserum dilution selected was that which bound 60% of the [ $^3\text{H}$ ]PG in the absence of any non-radioactive PG (zero standard). A 1:100 stock solution of antiserum in the appropriate diluent was used

to prepare the final dilution required.

Standard PG solutions were made up in diluent from a PG stock solution of  $1\mu\text{g/ml}$  in methanol;  $200\mu\text{l}$  of this stock solution were taken, and the methanol was evaporated off under a stream of air. The PG was redissolved in  $20\text{ml}$  of the appropriate diluent to give a final PG concentration of  $10\text{ng/ml}$ . Nine (for  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  assays) or ten (for  $\text{PGF}_{2\alpha}$  assays) standard solutions were prepared from this solution, ranging from  $0.02 - 5.12\text{ng PG/ml}$  for  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  standard solutions, and ranging from  $0.01 - 5.12\text{ng/ml}$  for  $\text{PGF}_{2\alpha}$  standard solutions. From each of these standard solutions,  $0.5\text{ml}$  was dispensed in triplicate into  $3\text{ml}$  plastic insert scintillation vials, the order of the tubes being as detailed in Table 2.3.1.i.

Counting standards and non-specific binding standards were also prepared and dispensed in quadruplicate as specified in Table 2.3.1.i. The four counting standards when averaged gave an estimate of the number of radioactive counts within each tube. The non-specific binding standards estimate  $[^3\text{H}]\text{PG}$  binding to sites other than the specific PG binding sites, which include the vial, non-specific sites on the antiserum, and other compounds used in the assay. The non-specific binding was always less than 10% in every assay.

The assay procedure is outlined in the flow diagram (Figure 2.3.1.a). Tissue culture samples and extracted samples in ethyl acetate were dispensed in duplicate into tubes using Eppendorf pipettes with disposable tips. Different volumes of the same sample (diluted if necessary) were prepared to ensure parallelism of results among volumes. It also ensured that the amount of PG measured was within the detection limits of the assay. Samples in ethyl acetate were taken to dryness at  $45^\circ\text{C}$ , under a stream of air. Diluent was

Table 2.3.1.i

The order and contents of the tubes containing the non-specific binding standards, counting standards, and PG standards when preparing a PG radioimmunoassay.

TUBE NUMBER	ng PG/TUBE		COMMENTS
1-4	5 ng		non-specific binding standards
5-8	-		counting standards, 50 $\mu$ l of the appropriate [ <sup>3</sup> H]PG solution per tube
9-11	0.01	0.005	PG standard curve
12-14	0.02	0.01	" " "
15-17	0.04	0.02	" " "
18-20	0.08	0.04	" " "
21-23	0.16	0.08	" " "
24-26	0.32	0.16	" " "
27-29	0.64	0.32	" " "
30-32	1.28	0.64	" " "
33-35	2.56	1.28	" " "
36-38		2.56	" " "

For assays measuring the amount of PG in tissue culture samples, 100 $\mu$ l culture medium were added to each PGF<sub>2</sub> $\alpha$  assay standard tube (total volume 600 $\mu$ l) or 200 $\mu$ l medium were added to each PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  assay standard tube (total volume 700 $\mu$ l). This ensures that the standard solutions and samples of tissue culture medium were comparable in their constituents.

Extracted samples:

Tissue culture medium samples:

dispense samples into tubes

dispense samples into tubes

↓  
evaporate off ethyl acetate to dryness in a stream of air at 45°C

↓  
add appropriate volume of diluent to maintain constant volume (600μl PGF<sub>2α</sub>; 700μl PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>) throughout assay

↘  
add 50μl appropriate [<sup>3</sup>H]PG  
add 50μl appropriate diluted PG antiserum

↓  
"whirlimix" and incubate at room temperature for 1h (PGF<sub>2α</sub> assays) or for 2h (PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> assays)

↓  
add 50μl NRS (1:100 dilution; not PGE<sub>2</sub> assay)  
add 50μl DARS (1:10 dilution)

↓  
"whirlimix" and incubate overnight at 4°C

↓  
centrifuge at 1300g for 30 min at 4°C

↓  
discard supernatant

↓  
add 3ml scintillation fluid and apply caps to vials

↓  
"whirlimix" to resuspend pellet

↓  
count each tube in liquid scintillation counter for 4 min (PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub>) or for 10 min (PGE<sub>2</sub>)

Figure 2.3.1.a

General protocol for PG radioimmunoassays.

then added to the dispensed samples; 500 $\mu$ l were added to extracted, dried residue samples, and an appropriate volume was added to the tissue culture medium samples to maintain constancy in the total volume (i.e. 600 $\mu$ l PGF<sub>2 $\alpha$</sub> ; 700 $\mu$ l PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub> ) throughout all the tubes within an assay (see Table 2.3.1.i.).

Tubes containing the samples and the standard solutions were then treated in the same way. To each tube were added 50 $\mu$ l of the appropriate [<sup>3</sup>H]PG solution and 50 $\mu$ l of the appropriate diluted PG antiserum. The tubes were "whirlmixed" and were then incubated at room temperature for 1h for PGF<sub>2 $\alpha$</sub>  assays or for 2h for PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  assays. Normal rabbit serum (NRS; 50 $\mu$ l; 1:100 dilution) and donkey anti-rabbit serum (DARS; 50 $\mu$ l; 1:10 dilution) were then added to each tube and the tubes "whirlmixed" again. NRS was added to ensure that, at high dilutions of the antiserum, there was sufficient gamma-globulin present for adequate precipitation subsequently. DARS was added to separate free PG from antibody-bound PG. However, it was not necessary to add NRS to the PGE<sub>2</sub> assay since a low dilution of PGE<sub>2</sub> antiserum was used. The tubes were incubated overnight at 4°C.

The following day, the tubes were centrifuged at 1300g for 30 min at 4°C. The supernatant was poured off and discarded, and 3ml scintillation fluid (section 2.1.2.v.) were dispensed into each tube. A cap was then inserted into the top of each tube, and the tubes were "whirlmixed" to resuspend the pellet. The tubes were scintillation counted for 4 min for PGF<sub>2 $\alpha$</sub>  and 6-keto-PGF<sub>1 $\alpha$</sub>  assays or for 10 min for PGE<sub>2</sub> assays using a Packard liquid scintillation counter.

Data from the counter were passed directly into an IBM-personal computer programmed with the Packard Data Acquisition and Analysis System (PC-DAAS). The Spline Curve-Fit programme was used which

processes the standards according to a Modified Smooth Spline Algorithm. This programme subtracted the amount of non-specific binding from all standards then calculated the co-ordinates for the curve of best-fit. Using this standard curve, the computer calculated the amount of PG in each sample tube. Sample tubes that lay outside the linear part of the curve were reassayed using different volumes or different dilutions of the sample.

The intra-assay coefficient of variation was calculated using the formula:

$$\text{intra-assay coefficient of variation} = \frac{\sum \left[ \frac{\text{standard deviation of sample duplicate}}{\text{mean value of sample duplicate}} \right]}{\text{total number of duplicates}} \times 100$$

Two tubes containing a known standard amount of the appropriate PG (0.16ng/tube) were included at the end of each assay, and the average amounts of PG measured in these assays were used to calculate the inter-assay coefficient of variation, using the formula:

$$\text{inter-assay coefficient of variation} = \frac{\text{standard deviation of average standard amount}}{\text{mean of average standard amount}} \times 100$$

Cross-reactivities for each of the PG antisera have been determined previously in this laboratory. The method used entails the setting up of a standard curve for the PG together with standard curves for the different prostaglandins and PG metabolites. The concentration of the different PG or PG metabolite which produced a 50% fall in binding from the binding produced by a zero standard solution of the PG to which the antibodies were raised was obtained and the percentage cross-reactivity was calculated using the formula:



$$\% \text{ cross-reactivity} = \frac{\text{concentration of the PG (to which the antisera was raised) giving a 50\% fall in zero standard binding}}{\text{concentration of different PG or PG metabolite giving a 50\% fall in zero standard binding}} \times 100$$

### 2.3.2 Measurement of PGF<sub>2α</sub> by radioimmunoassay

A standard curve for PGF<sub>2α</sub> was set up as detailed in Table 2.3.1.i. The general assay protocol was followed as given in section 2.3.1, using the variations for the PGF<sub>2α</sub> radioimmunoassay as outlined there. The reagents used were:

Tris diluent pH 8

[<sup>3</sup>H]PGF<sub>2α</sub> (50μl of a 0.125 μCi/ml solution)

PGF<sub>2α</sub> antiserum (rabbit 6, 6th bleed), 50μl of a 1:1200 dilution (i.e. 1:1200)

#### Results

The mean (± s.e.m.) values of the standard curves from six consecutive PGF<sub>2α</sub> assays are shown in Figure 2.3.2.a.

Intra-assay coefficient of variation = 5.6%

Inter-assay coefficient of variation = 8.2%

Table 2.3.2.i. shows the cross-reactivities of the PGF<sub>2α</sub> antiserum (rabbit 6, 6th bleed) with other prostaglandins and their metabolites as determined previously (Dighe, Emslie, Henderson, Rutherford and Simon, 1975; Poyser and Scott, 1980; Lytton and Poyser, 1982).

#### Conclusion

The PGF<sub>2α</sub> antiserum cross-reacts with PGF<sub>1α</sub> to a significant extent. Previous work by Poyser (1983a) analysing PGs produced by the guinea-pig uterus by gas chromatography-mass spectrometry (GC-MS) showed that very little PGF<sub>1α</sub> was synthesised in comparison with

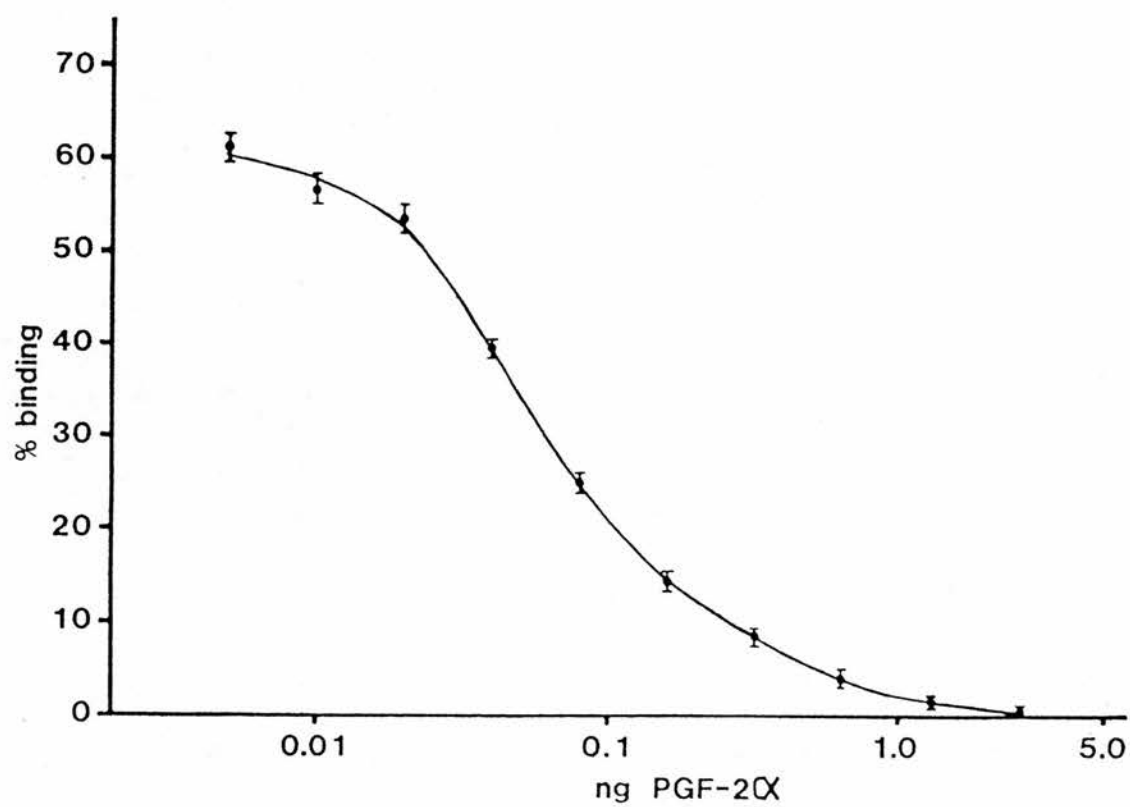


Figure 2.3.2.a

Standard curve for PGF<sub>2</sub>α radioimmunoassay (mean ± s.e.m., n = 6).

Table 2.3.2.i

Cross-reactivities with various prostanoids and their metabolites of the  $\text{PGF}_{2\alpha}$  antiserum (rabbit 6, 6th bleed), measured at 30% binding of tracer.

PROSTANOID	% CROSS-REACTIVITY AT 30% BINDING OF TRACER
$\text{PGF}_{2\alpha}$	100
$\text{PGF}_{1\alpha}$	28.0
$\text{PGE}_2$	0.2
$\text{PGE}_1$	0.5
$\text{PGA}_2$	< 0.1
$\text{PGB}_2$	< 0.1
$\text{PGD}_2$	0.4
15-keto- $\text{PGF}_{2\alpha}$	0.5
13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$	0.2
6-keto- $\text{PGF}_{1\alpha}$	0.5
15-keto- $\text{PGE}_2$	< 0.1
13,14-dihydro-15-keto- $\text{PGE}_2$	0.1
$\text{TXB}_2$	0.6

PGF<sub>2α</sub>. Therefore, in these studies it is highly probable that the PGF<sub>2α</sub> antiserum was measuring predominantly the PG to which it had been raised.

### 2.3.3 Measurement of PGE<sub>2</sub> by radioimmunoassay

A standard curve for PGE<sub>2</sub> was prepared as detailed in Table 2.3.1.i. The general assay procedure was followed (Section 2.3.1) using the variations for the PGE<sub>2</sub> as outlined previously. The reagents used were:

Phosphate diluent pH 7.5

[<sup>3</sup>H]PGE<sub>2</sub> (50μl of a 0.063μCi/ml solution)

PGE<sub>2</sub> antiserum (rabbit 5, 6th bleed), 50μl of a 1:300 dilution

#### Results

Figure 2.3.3.a shows the combined results (mean ± s.e.m.) of the standard curves from six consecutive PGE<sub>2</sub> radioimmunoassays.

Intra-assay coefficient of variation = 7.8%

Inter-assay coefficient of variation = 8.6%

Table 2.3.3.i shows the cross-reactivities of the PGE<sub>2</sub> antiserum (rabbit 5, 6th bleed) with other prostanoids and their metabolites as determined previously (Poyser and Scott, 1980; Lytton and Poyser, 1982).

#### Conclusions

The PGE<sub>2</sub> antibody showed a high cross-reactivity with PGE<sub>1</sub> and PGB<sub>2</sub> and some cross-reactivity with PGA<sub>2</sub>. Previous work which involved analysing the PGs produced by the guinea-pig uterus by GC-MS showed that PGE<sub>1</sub>, PGA<sub>2</sub> and PGB<sub>2</sub> were not synthesised in any detectable quantities (Poyser, 1983a), so the measurements made using this PGE<sub>2</sub> antiserum are very probably of PGE<sub>2</sub>.

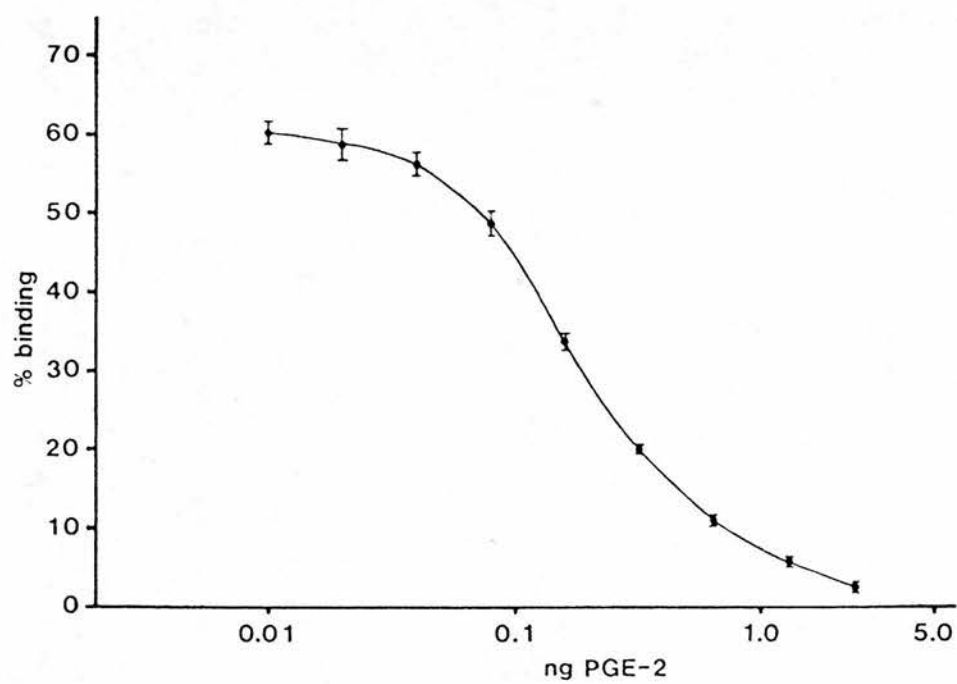


Figure 2.3.3.a

Standard curve for PGE<sub>2</sub> radioimmunoassay (mean ± s.e.m., n = 6).

Table 2.3.3.i

Cross-reactivities with various prostanoids and their metabolites of the PGE<sub>2</sub> antiserum (rabbit 5, 6th bleed), measured at 30% binding of tracer.

PROSTANOID	% CROSS-REACTIVITY AT 30% BINDING OF TRACER
PGE <sub>2</sub>	100
PGE <sub>1</sub>	94.0
PGA <sub>2</sub>	13.6
PGB <sub>2</sub>	72.7
PGD <sub>2</sub>	0.4
PGF <sub>1α</sub>	4.3
PGF <sub>2α</sub>	3.8
6-keto-PGF <sub>1α</sub>	0.3
15-keto-PGF <sub>2α</sub>	0.1
13,14-dihydro-15-keto-PGE <sub>2α</sub>	< 0.1
15-keto-PGE <sub>2</sub>	0.2
13,14-dihydro-15-keto-PGE <sub>2</sub>	0.4
TXB <sub>2</sub>	0.2

#### 2.3.4 Measurement of 6-keto-PGF<sub>1α</sub> by radioimmunoassay

A standard curve for 6-keto-PGF<sub>1α</sub> was prepared as detailed in Table 2.3.1.i. The general assay protocol was followed (section 2.3.1) using the specific changes for this assay as described. The reagents used were:

Tris diluent pH 6.8

[<sup>3</sup>H]6-keto-PGF<sub>1α</sub> (50μl of a 0.2 μCi/ml solution)

6-keto-PGF<sub>1α</sub> antiserum (rabbit NP 1, 6th bleed) 50μl 1:1400 dilution.

##### Results

The mean ( $\pm$  s.e.m.) values of the standard curves of six consecutive 6-keto-PGF<sub>1α</sub> assays are shown in Figure 2.3.4.a.

Intra-assay coefficient of variation = 5.9%

Inter-assay coefficient of variation = 6.6%

Table 2.3.4.i shows the cross-reactivities of the 6-keto-PGF<sub>1α</sub> antiserum used with other prostanoids as determined previously (Poyser, 1980; Poyser and Scott, 1980).

#### 2.3.5 Measurement of progesterone by radioimmunoassay

##### Introduction

This assay was used to measure the progesterone concentration in samples of plasma removed from guinea-pigs. After stunning the guinea-pig and incising the neck, a blood sample (5ml) was taken from the incised neck into a syringe containing heparin (1000U/ml; 0.1ml). The blood sample was then centrifuged (2500g) and the plasma was removed and stored at -20°C. Progesterone was extracted just prior to assaying the samples. The protocol of the progesterone radioimmunoassay was similar to that of the PG radioimmunoassay, so it will only be described in brief. This radioimmunoassay was adapted

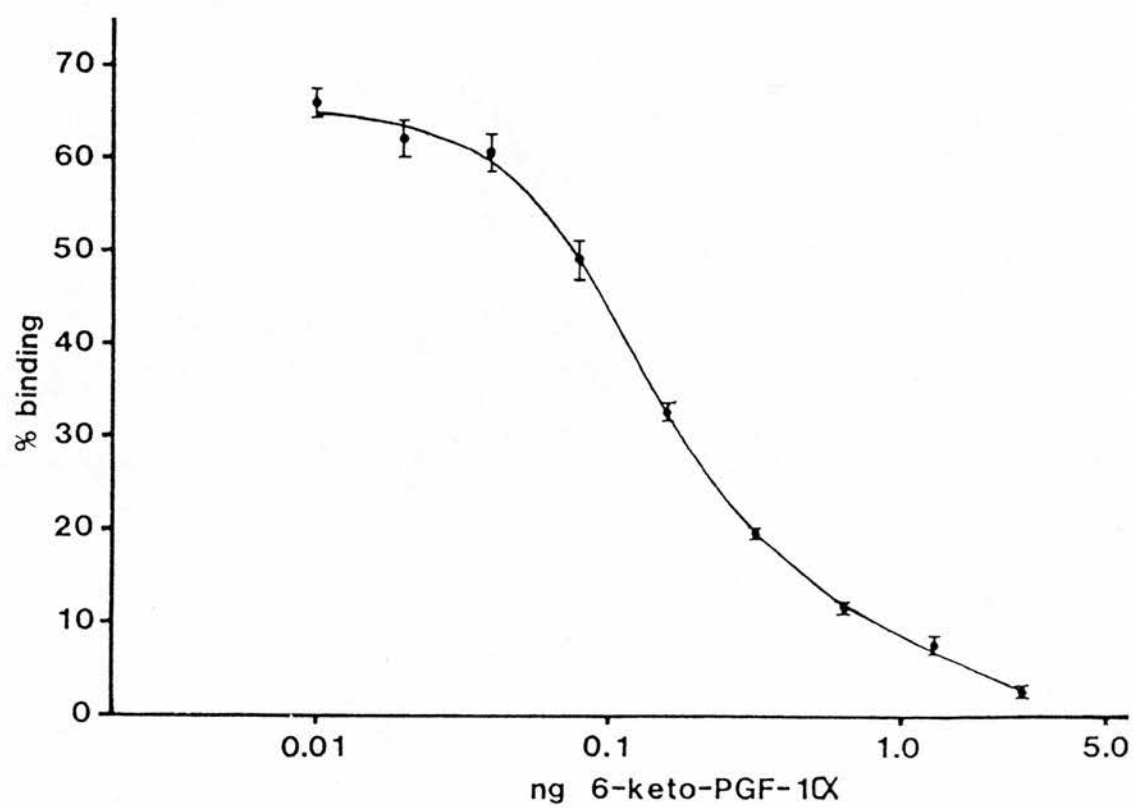


Figure 2.3.4.a

Standard curve for 6-keto-PGF<sub>1</sub>α radioimmunoassay (mean ± s.e.m.,  
n = 6).



Table 2.3.4.i

Cross-reactivities with various prostanoids and their metabolites of the 6-keto-PGF<sub>1α</sub> antiserum (rabbit 1, 6th bleed), measured at 30% binding of tracer.

PROSTANOID	% CROSS-REACTIVITY AT 30% BINDING OF TRACER
6-keto-PGF <sub>1α</sub>	100
PGA <sub>2</sub>	< 0.1
PGB <sub>2</sub>	< 0.1
PGD <sub>2</sub>	< 0.1
PGE <sub>1</sub>	1.1
PGE <sub>2</sub>	4.2
PGF <sub>1α</sub>	0.4
PGF <sub>2α</sub>	< 0.1
15-keto-PGF <sub>2α</sub>	< 0.1
13,14-dihydro-15-keto-PGF <sub>2α</sub>	0.1
15-keto-PGE <sub>2</sub>	0.1
13,14-dihydro-15-keto-PGE <sub>2</sub>	0.1
TXB <sub>2</sub>	< 0.1

from the method described previously by Poyser and Horton (1975).

#### Extraction of progesterone from plasma samples

Progesterone was extracted from samples by the method described by Dighe and Hunter (1974). The samples were dispensed out in two volumes in duplicate into glass test tubes and 2ml redistilled petroleum spirit (b.pt. 40-60°C) were added. The tubes were shaken vigorously for 3 min in a Baird and Tatlock multivortex shaker. the lower aqueous layer was frozen in a bath of acetone containing dry ice. The upper layer was decanted into a small glass test tube. This extraction process was then repeated. The petroleum spirit extract was blown to dryness with a jet of air, at 60°C.

#### Antiserum used

Antiserum for progesterone was raised in rabbits by immunising them with progesterone-11 $\alpha$ -hemisuccinate-bovine serum albumin, as described by Poyser (1983a) using the method of Dighe and Hunter (1974).

#### Reagents

Phosphate diluent pH 7.5

[<sup>3</sup>H]progesterone (50 $\mu$ l of a 0.036  $\mu$ Ci/ml solution)

progesterone antiserum (Rabbit NP 3, 2nd bleed) 50 $\mu$ l of a 1:600 dilution.

#### Method

From a stock solution of 1 $\mu$ g/ml progesterone in methanol the standard solutions were prepared. Initially, 200 $\mu$ l of this stock solution were taken, and the methanol was blown off and 20ml phosphate diluent pH 7.5 were added. Nine standard solutions ranging from 0.02ng/ml to 5.12ng/ml were prepared from this solution (10ng/ml). Each standard solution was dispensed into glass test tubes in triplicate (1ml each standard solution per tube), with counting

standards, non-specific binding standards and zero standards all in quadruplicate, as detailed in Table 2.3.5.i. The % binding of tracer by the zero standards indicates the maximum binding of the tracer by the added antiserum in the absence of non-radioactive progesterone. The remainder of the protocol for preparing the assay is detailed in Figure 2.3.5.a., and is similar to the PG radioimmunoassay protocol (section 2.3.1).

However, this progesterone radioimmunoassay differs from the PG radioimmunoassays because supernatant containing free [ $^3\text{H}$ ]progesterone was counted in the assay tubes by the scintillation counter rather than counting the bound [ $^3\text{H}$ ]progesterone in the pellet fraction. The vials were counted for 10 min in a Nuclear Chicago Liquid Scintillation counter. The results for the standard curve were processed by a Commodore 4032 computer programmed to subtract background counts and then calculate the percentage of bound [ $^3\text{H}$ ]progesterone from the amount of free [ $^3\text{H}$ ]progesterone present in the supernatant, according to the formula:

$$\% \text{ bound} = 100 - \frac{\text{sample counts} - \text{background counts}}{\text{average counting standards} - \text{background counts}} \times 100$$

The standard curve was plotted and the line of best fit was drawn by hand. The percentage of [ $^3\text{H}$ ]progesterone bound was calculated for the extracted plasma samples, and compared with the standard curve. The amount of progesterone per sample was then measured from the standard curve.

### Results

Figure 2.3.5.b shows the mean ( $\pm$  s.e.m.) results of the standard

Table 2.3.5.i

The order and contents of the tubes containing the non-specific binding standards, counting standards, zero standards and progesterone standards when preparing a progesterone radioimmunoassay.

TUBE NUMBER	ng progesterone/ tube	COMMENTS
1-4	10	non-specific binding standards
5-8	-	counting standards, 50 $\mu$ l of [ $^3$ H] progesterone solution
9-12	0	zero standards
13-15	5.12	progesterone standard curve
16-18	2.56	" " "
19-21	1.28	" " "
22-24	0.64	" " "
25-27	0.32	" " "
28-30	0.16	" " "
31-33	0.08	" " "
34-36	0.04	" " "
37-39	0.02	" " "

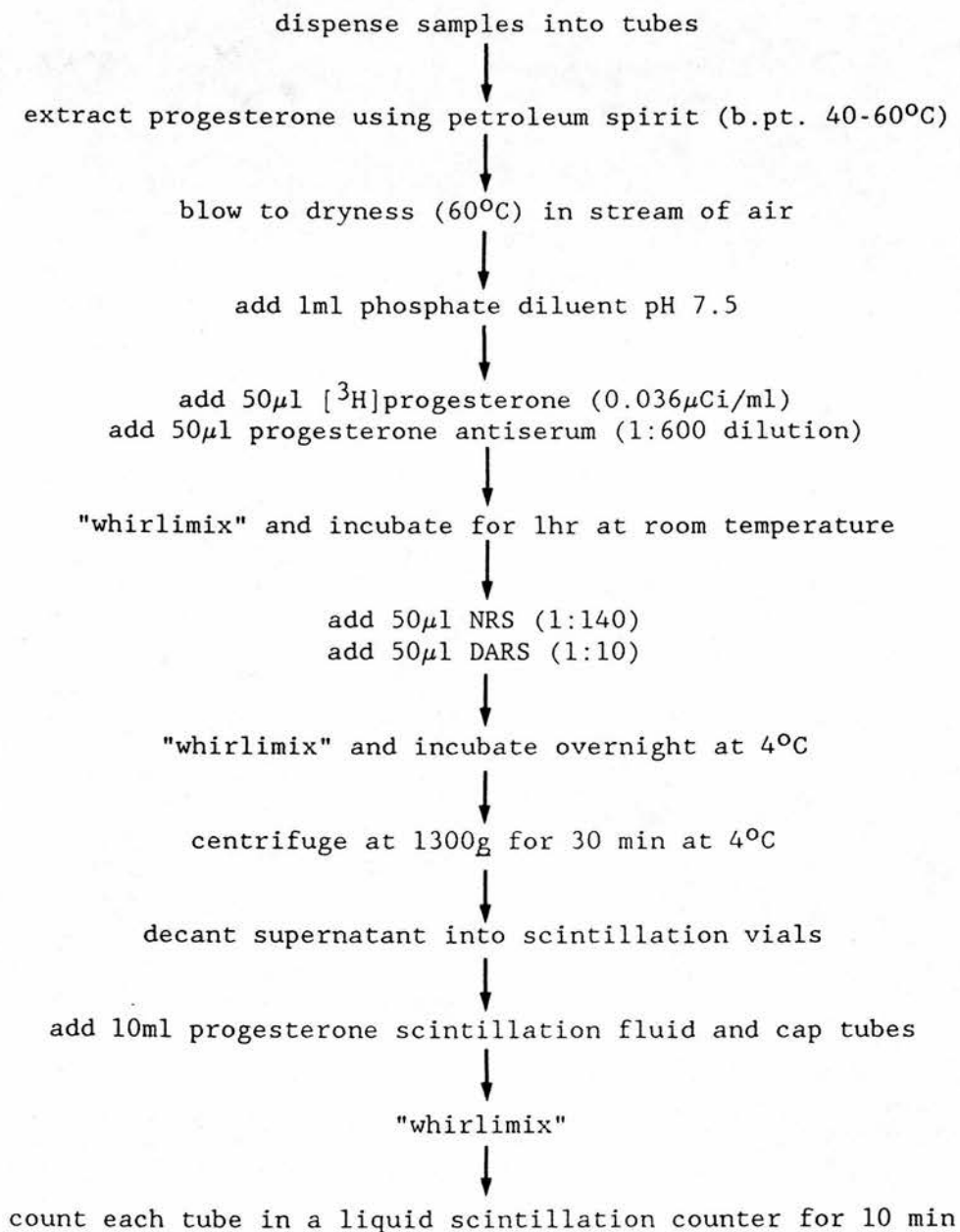


Figure 2.3.5.a

Protocol for setting up a progesterone radioimmunoassay

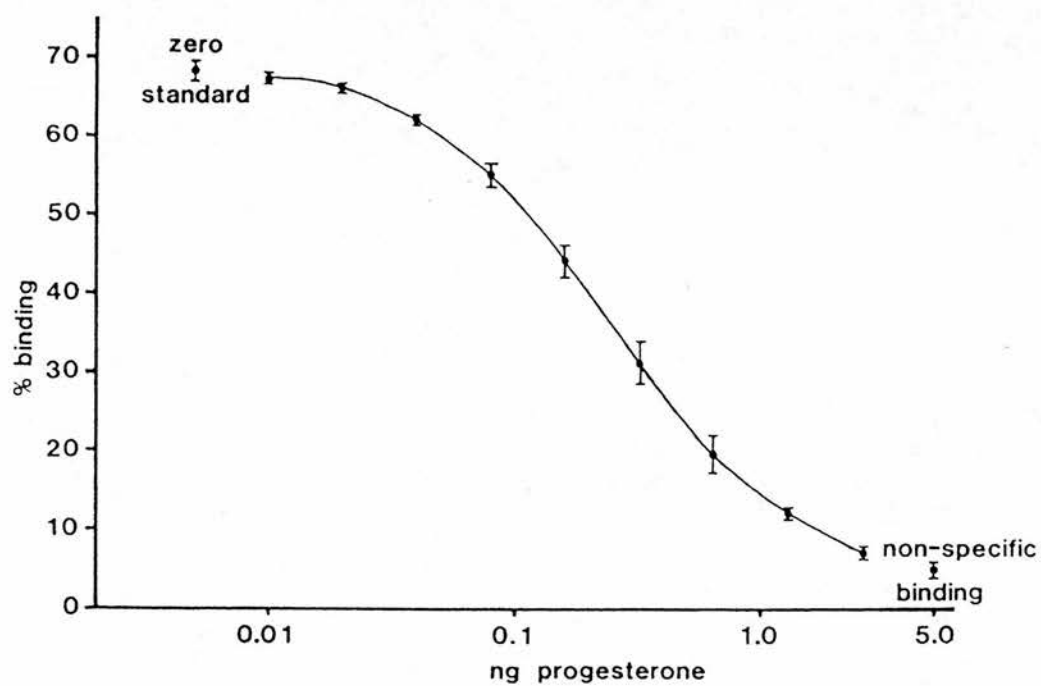


Figure 2.3.5.b

Standard curve for progesterone radioimmunoassay (mean  $\pm$  s.e.m.,  $n = 6$ ).

Table 2.3.5.ii

Cross-reactivities with various compounds of the progesterone antiserum (rabbit NP 13, 2nd bleed), measured at 30% binding of tracer.

COMPOUND	% CROSS-REACTIVITY AT 30% BINDING OF TRACER
Progesterone	100
11 $\alpha$ -hydroxyprogesterone	37.5
11 $\beta$ -hydroxyprogesterone	29.2
17 $\alpha$ -hydroxyprogesterone	2.0
Oestradiol-17 $\beta$	0.6
Oestriol	0.1
Oestrone	< 0.1
Cortisone	1.5
hydrocortisone	0.3
cholesterol	0.2
testosterone	0.1
20 $\alpha$ -hydroxy- $\Delta^4$ -pregnen-3-one	1.4
20 $\beta$ -hydroxy-3 $\beta$ -ol-20-one	1.9
$\Delta^5$ -pregnen-3 $\beta$ -ol-20-one	1.4
$\Delta^4$ -androstene-3,17-dione	0.4

curves of six consecutive progesterone assays.

Intra-assay coefficient of variation = 10.8%

Inter-assay coefficient of variation = 10.1%

Table 2.3.5.ii shows the cross-reactivities of the progesterone antiserum (Rabbit NP 13, 2nd bleed) at 50% binding of the tracer as determined by the method of Dighe and Hunter (1974). The cross reactivities have been reported previously by Poyser (1983a; 1984b).

#### Conclusion

The progesterone antiserum showed a high cross-reactivity with  $11\alpha$ -hydroxyprogesterone and  $11\beta$ -hydroxyprogesterone. However, these are synthetic steroids and are not present in vivo. The values for plasma progesterone concentrations obtained by this assay and which are reported later (see section 3.5) were in the expected range (Challis, Heap and Illingworth, 1971; Poyser and Horton, 1975). Plasma progesterone concentrations declined before oestrus (see section 3.5). Therefore the antiserum was regarded as suitable to measure guinea-pig plasma progesterone levels.

### 2.4 MEASUREMENT OF ENDOMETRIAL PROTEIN SYNTHESIS

#### Introduction

This technique was used to investigate protein synthesis by pieces of the guinea-pig endometrium maintained in tissue culture, by measuring [ $^3\text{H}$ ]-leucine incorporation into both cellular and secreted proteins.

The technique used to measure synthesis of cellular proteins was adapted from the method of Findlay, Ackland, Burton, Davis, Maule Walker, Walters and Heap (1981). Synthesis of secretory proteins was measured by an adaptation of the method described by Strinden and Shapiro (1983). Endometrium was prepared for tissue culture as



explained in section 2.2.5, with 30-60mg tissue being placed in each dish. The medium of each dish contained 10 $\mu$ Ci [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml), and the tissue was incubated for up to 24h under conditions previously described (section 2.2.5). Further supplements to the medium and incubation times are given later (see sections 3.4, 3.5 and 3.6). The methods for measuring [ $^3$ H]-leucine incorporation into cellular and secreted proteins are outlined in Figure 2.4.a.

#### 2.4.1 Measurement of [ $^3$ H]leucine incorporated into cellular proteins

The tissue was weighed after blotting dry, placed in a 3ml scintillation tube and washed three times with 1ml Medium 199 at 4°C. Lysis buffer (1ml; 8M urea, 1% v/v sodium dodecyl sulphate and 5% v/v mercaptoethanol) was added, the cap was inserted into the top of the tube, and the tube was shaken and then incubated at 4°C overnight. Tissue protein was extracted by freezing on solid CO<sub>2</sub>, then allowing to thaw at room temperature while "whirlmixing". This freeze-thaw process was carried out three times. After centrifugation at 1400g for 15 min, 200 $\mu$ l supernatant were added in quadruplicate to 300 $\mu$ l saline (0.9g/ml) containing bovine serum albumin (BSA; 0.1% w/v) and L-leucine (0.1% w/v). Protein was precipitated by adding 500 $\mu$ l 10% w/v trichloroacetic acid (TCA), and the tubes were "whirlmixed" and then incubated for 30 min at 4°C. The tubes were centrifuged at 1400g for 15 min and washed once with 500 $\mu$ l 5% w/v TCA, "whirlmixed" to resuspend the precipitate and centrifuged at 1400g for 15 min. The supernatant was discarded and the precipitate was dissolved by incubating with 100 $\mu$ l formic acid for 30 min at room temperature. Scintillation fluid (3ml; see section 2.1.2.v) was added, caps were inserted into the tops of tubes, and the tubes were counted in a Packard liquid scintillation counter for 10 min.

#### 2.4.2 Measurement of [<sup>3</sup>H]-leucine incorporation into secreted proteins

The culture medium was centrifuged in glass tubes at 1000g for 10 min. Four 200 $\mu$ l aliquots of the supernatant were placed in 3ml scintillation vials and BSA solution (in saline) was added (10 $\mu$ l of a 0.2g BSA/10ml saline solution; 0.1% w/v solution). Protein was precipitated with 300 $\mu$ l 10% w/v TCA, and the tubes were "whirlmixed" and incubated for 30 min at 4°C. The tubes were centrifuged at 1400g for 15 min at 4°C and then the supernatant was discarded. The precipitate was washed with 300 $\mu$ l 5% w/v TCA, "whirlmixed" and centrifuged again. This wash process to remove any free [<sup>3</sup>H]-leucine was carried out 3 times. Finally, the supernatant was discarded, the precipitate was incubated for 30 min with 100 $\mu$ l formic acid to dissolve the pellet, 3ml scintillation fluid (see section 2.1.2.v) were added to each tube, caps were inserted into the tops of each tube, and then the tubes were counted in a Packard liquid scintillation counter for 10 min.

Four counting standard tubes were prepared consisting of 50 $\mu$ l culture medium containing [<sup>3</sup>H]-leucine (2.5 $\mu$ Ci/ml; retained after dispensing the medium into the Petri dishes), 100 $\mu$ l formic acid and 3ml scintillation fluid. Also, four background counting tubes were prepared containing 100 $\mu$ l formic acid and 3ml scintillation fluid. These tubes were counted for 10 min in the liquid scintillation counter with the sample tubes.

Cellular proteins:

measure wet weight of tissue  
↓  
wash 3 times with culture medium  
↓  
add 1ml lysis buffer, shake and leave overnight  
↓  
extract tissue protein by freezing on solid CO<sub>2</sub> and thawing and "whirlmixing" 3 times  
↓  
centrifuge (1400g for 15 min)  
↓  
take 200μl supernatant and add 300μl saline (containing BSA and L-leucine supplements) and 500μl 10% w/v TCA  
↓  
"whirlmix" and leave for 30 min at 4°C  
↓  
centrifuge (1400g for 15 min)  
↓  
discard supernatant and wash pellet once with 500μl 5% w/v TCA and "whirlmix"  
↓  
centrifuge (1400g for 15 min)  
↓  
discard supernatant

Secreted proteins:

centrifuge medium (100g for 10 min)  
↓  
take 200μl supernatant and add 10μl BSA solution (0.1% w/v solution)  
Then add 300μl 10% w/v TCA.  
↓  
"whirlmix" and leave for 30 min at 4°C  
↓  
centrifuge (1400g for 15 min)  
↓  
discard supernatant, add 300μl 5% w/v TCA and "whirlmix"  
↓  
centrifuge (1400g for 15 min)  
↓  
wash, "whirlmix" and centrifuge 2 more times  
↓  
discard supernatant

↓  
dissolve pellet in 100μl formic acid and leave for 30 min  
↓  
add 3ml scintillation fluid  
↓  
liquid scintillation count for 10 min

Figure 2.4.a

The methods used for measuring [<sup>3</sup>H]-leucine incorporation into cellular and secreted proteins.

## SECTION 3

### RESULTS

#### 3.1 PROSTAGLANDIN OUTPUT FROM THE GUINEA-PIG ENDOMETRIUM IN CULTURE

##### INTRODUCTION

PGF<sub>2α</sub> is the uterine luteolytic hormone in the guinea-pig. The output of PGF<sub>2α</sub> from the uterus increased from day 11 to the end of the oestrous cycle when measured in the utero-ovarian vein (Blatchley, Donovan, Horton and Poyser, 1972). Poyser and Brydon (1983) have shown that PGF<sub>2α</sub> output, when measured from the superfused guinea-pig uterus in vitro, increases 21.9-fold between day 7 and day 15 of the cycle, and Poyser (1983a) showed that the primary site of PGF<sub>2α</sub> synthesis is the endometrium. Leaver and Seawright (1982) examined the output of PGF<sub>2α</sub> from guinea-pig endometrium cultured for three days and showed that PGF<sub>2α</sub> output was greater on day 15 than on day 7 and that output declined with time.

The culturing of guinea-pig endometrium, removed on day 7 and day 15 of the oestrous cycle, has been one of the major experimental techniques used in the studies presented in this thesis. The aims of this experiment are:

- (a) to examine and compare the outputs of PGF<sub>2α</sub>, and also of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> which have not previously been measured, from guinea-pig endometrium removed on day 7 and day 15 of the oestrous cycle and cultured for three days.
- (b) to conduct a more detailed analysis of the outputs of these three PGs from guinea-pig endometrium removed on day 7 and day 15 of the oestrous cycle and sampled at 6 hourly intervals over the initial 24h period of culture.
- (c) to establish the outputs of these PGs from guinea-pig endometrium removed on day 7 and day 15 of the oestrous cycle and

culture for three days and for 24h in order to allow comparison with various treatments in the following experimental results sections 3.2, 3.3, 3.4, 3.5 and 3.6.

## METHODS

Virgin female guinea-pigs were used on day 7 and day 15 of the oestrous cycle. The animals were killed and their uteri were removed in preparation for tissue culture (section 2.2.1). The endometrium was dissected from the myometrium, and between 10 and 20 mg endometrium in small pieces, were placed in each petri-dish for culturing as explained in section 2.2.5. Four dishes containing endometrium from each uterus were prepared with two dishes being cultured in each of two modified Kilner jars which were gassed and incubated as explained in section 2.2.5. The frequency of changing the medium is explained in the specific experimental methods below. All samples of culture medium were stored at  $-20^{\circ}\text{C}$  before radioimmunoassay, without extraction, for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (see section 2.3).

### Experiment 1: Basal PG output from day 7 and day 15 guinea-pig endometrium cultured for three days

Uteri were removed from five day 7 and five day 15 guinea-pigs. Endometrium from each uterus was removed and cultured for 72h as explained above. The medium was changed every 24h and the jars were regassed before being replaced in the incubator.

### Experiment 2: Basal PG output from day 7 and day 15 guinea-pig endometrium cultured for 24h

Uteri from five day 7 and five day 15 guinea-pigs were used. Endometrium from each uterus was cultured for 24h, as explained above. The medium was changed every 6h, and the jars containing the dishes

were regassed after every change before being returned to the incubator.

#### Statistical tests

Student's 't' test (or the modified 't' test if the variances were unequal as shown by the variance ratio F-test) was used to compare day 7 and day 15 outputs for the same PG at the same time. Duncan's multiple-range test was used in Experiment 2 to analyse changes in the outputs of PGs into the culture medium with time.

### RESULTS

#### Experiment 1: Basal PG output from day 7 and day 15 guinea-pig endometrium cultured for three days

The outputs from day 7 and day 15 guinea-pig endometrium of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  significantly ( $P < 0.05$ ) declined from day 1 to day 3 of culture (Figure 3.1.a). The ratios of the mean PG outputs from day 15 compared to day 7 endometrium on days 1, 2 and 3 of culture, for  $\text{PGF}_{2\alpha}$  were 7.2:1.0, 3.3:1.0 and 2.1:1.0, respectively, for  $\text{PGE}_2$  were 3.3:1.0, 2.4:1.0 and 1.3:1.0, respectively, and for 6-keto- $\text{PGF}_{1\alpha}$  were 1.2:1.0, 1.3:1.0 and 1.2:1.0, respectively. The output of  $\text{PGF}_{2\alpha}$  was significantly ( $P < 0.05$ ) higher from day 15 endometrium than from day 7 endometrium on all three days of culture. The output of  $\text{PGE}_2$  was significantly ( $P < 0.05$ ) higher from day 15 than from day 7 endometrium during days 1 and 2 of culture, and the output of 6-keto- $\text{PGF}_{1\alpha}$  was significantly ( $P < 0.05$ ) higher from day 15 than from day 7 endometrium only during day 2 of culture. The ratios of the mean outputs of  $\text{PGF}_{2\alpha}$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{PGE}_2$  on days 1, 2 and 3 of culture for day 7 cultured endometrium were 7.3:11.0:1.0, 9.8:4.8:1.0 and 6.7:4.3:1.0, respectively, and for day 15 cultured tissue they were 13.9:3.4:1.0, 13.4:2.6:1.0 and 8.2:3.2:1.0,

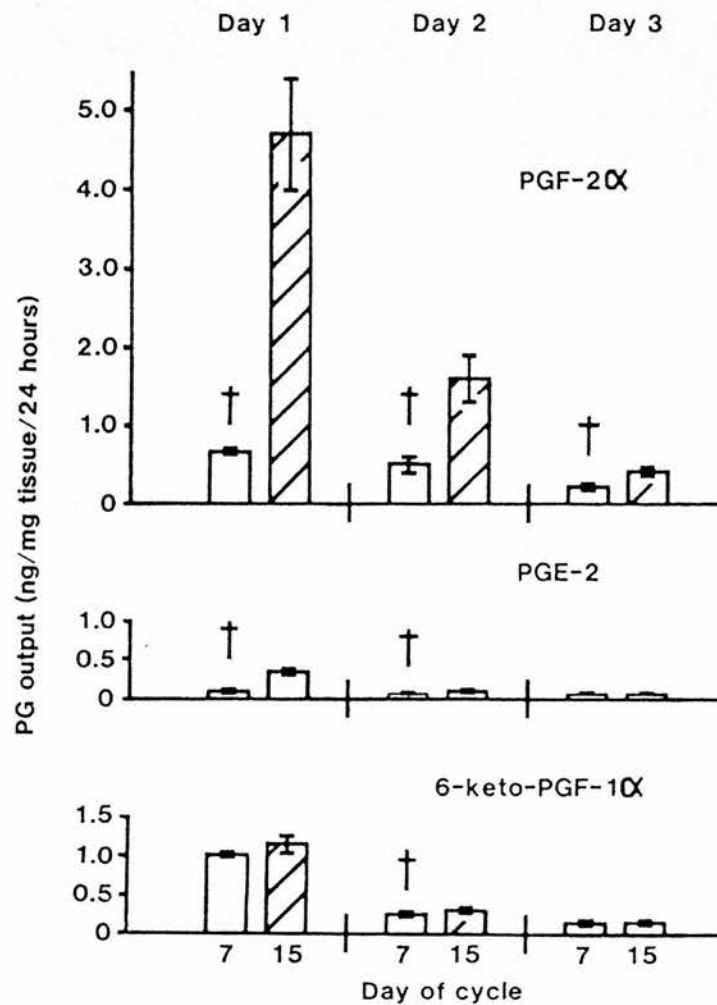


Figure 3.1.a

Mean (± s.e.m.; n = 10) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α from guinea-pig endometrium removed on day 7 (open columns) or on day 15 (hatched columns) of the oestrous cycle and cultured for three days, with sampling every 24h.

† output significantly (P < 0.05) lower compared to day 15 output for the same PG on the same day of culture.

respectively. The outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were similar from day 7 endometrium with a lesser amount of  $\text{PGE}_2$  being released. However,  $\text{PGF}_{2\alpha}$  was the major PG produced from day 15 endometrium, with lesser quantities of 6-keto- $\text{PGF}_{1\alpha}$  and even smaller amounts of  $\text{PGE}_2$  being released.

Experiment 2: Basal PG output from day 7 and day 15 guinea-pig endometrium cultured for 24h

The output of  $\text{PGF}_{2\alpha}$  from day 7 endometrium significantly ( $P < 0.05$ ) increased during the first 18h of culture, then declined slightly during the final sample period of 18 to 24h (Figure 3.1.b). The output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium significantly ( $P < 0.05$ ) declined during 24h in culture (Figure 3.1.b).  $\text{PGE}_2$  output from day 7 endometrium did not change significantly throughout the culture period, whereas  $\text{PGE}_2$  output from day 15 endometrium significantly ( $P < 0.05$ ) declined over 24h in culture (Figure 3.1.b). The output of 6-keto- $\text{PGF}_{1\alpha}$  from both day 7 and day 15 guinea-pig endometrium significantly ( $P < 0.05$ ) declined during the 24h period of culture (Figure 3.1.b). The ratios of the mean PG outputs from day 15 compared to day 7 tissue, between 0h and 6h, 6h and 12h, 12h and 18h and 18h and 24h of culture, for  $\text{PGF}_{2\alpha}$  were 26.5:1.0, 9.8:1.0, 3.0:1.0 and 2.5:1.0, respectively, for  $\text{PGE}_2$  were 4.5:1.0, 4.9:1.0, 1.8:1.0 and 1.8:1.0, respectively, and for 6-keto- $\text{PGF}_{1\alpha}$  were 0.9:1.0, 1.5:1.0, 1.1:1.0 and 0.9:1.0, respectively. The outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from day 15 endometrium were significantly ( $P < 0.05$ ) higher than from day 7 endometrium during all the sample periods over 24h. The output of 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium was significantly ( $P < 0.05$ ) higher than from day 7 endometrium only between 6h and 12h. The ratios of the mean outputs of  $\text{PGF}_{2\alpha}$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{PGE}_2$  between 0 and 6h, 6h and 12h, 12h and 18h, and 18h and 24h of culture from



endometrium of day 7 animals were 2.3:14.7:1.0, 8.0:9.9:1.0, 9.7:3.5:1.0 and 8.2:3.1:1.0, respectively. For day 15 endometrium these ratios were 13.3:2.8:1.0, 15.9:3.1:1.0, 15.8:2.2:1.0 and 11.8:1.6:1.0, respectively. During the first two sample periods, 6-keto-PGF<sub>1α</sub> output from day 7 endometrium was significantly ( $P < 0.05$ ) greater than PGF<sub>2α</sub> output. However, for the period between 12h and 24h, PGF<sub>2α</sub> output was significantly ( $P < 0.05$ ) greater than 6-keto-PGF<sub>1α</sub> output. The output of PGE<sub>2</sub> was lower than the outputs of PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub>. More PGF<sub>2α</sub> than PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> was released from day 15 endometrium. The amount of 6-keto-PGF<sub>1α</sub> released was more than the amount of PGE<sub>2</sub> released from day 15 endometrium.

### CONCLUSIONS

The basal outputs of PGF<sub>2α</sub> and, to a lesser degree, PGE<sub>2</sub> from guinea-pig endometrium cultured for three days or for 24h were higher from tissue removed on day 15 than from tissue removed on day 7 of the oestrous cycle. The output of 6-keto-PGF<sub>1α</sub> from endometrium was similar on both day 7 and 15 for three days and for 24h of culture. From both day 7 and day 15 endometrium cultured for three days, the output of all three PGs declined with time. During the first 24h of culture, the outputs of all three PGs from day 15 endometrium and the output of 6-keto-PGF<sub>1α</sub> from day 7 tissue also declined. However, PGF<sub>2α</sub> output from day 7 tissue cultured for 24h increased to a peak between 12h and 18h. The output of PGE<sub>2</sub> from day 7 endometrium did not significantly change from the output during the first 6h of culture throughout the culture period.

### DISCUSSION

The higher output of PGF<sub>2α</sub> from day 15 when compared to day 7

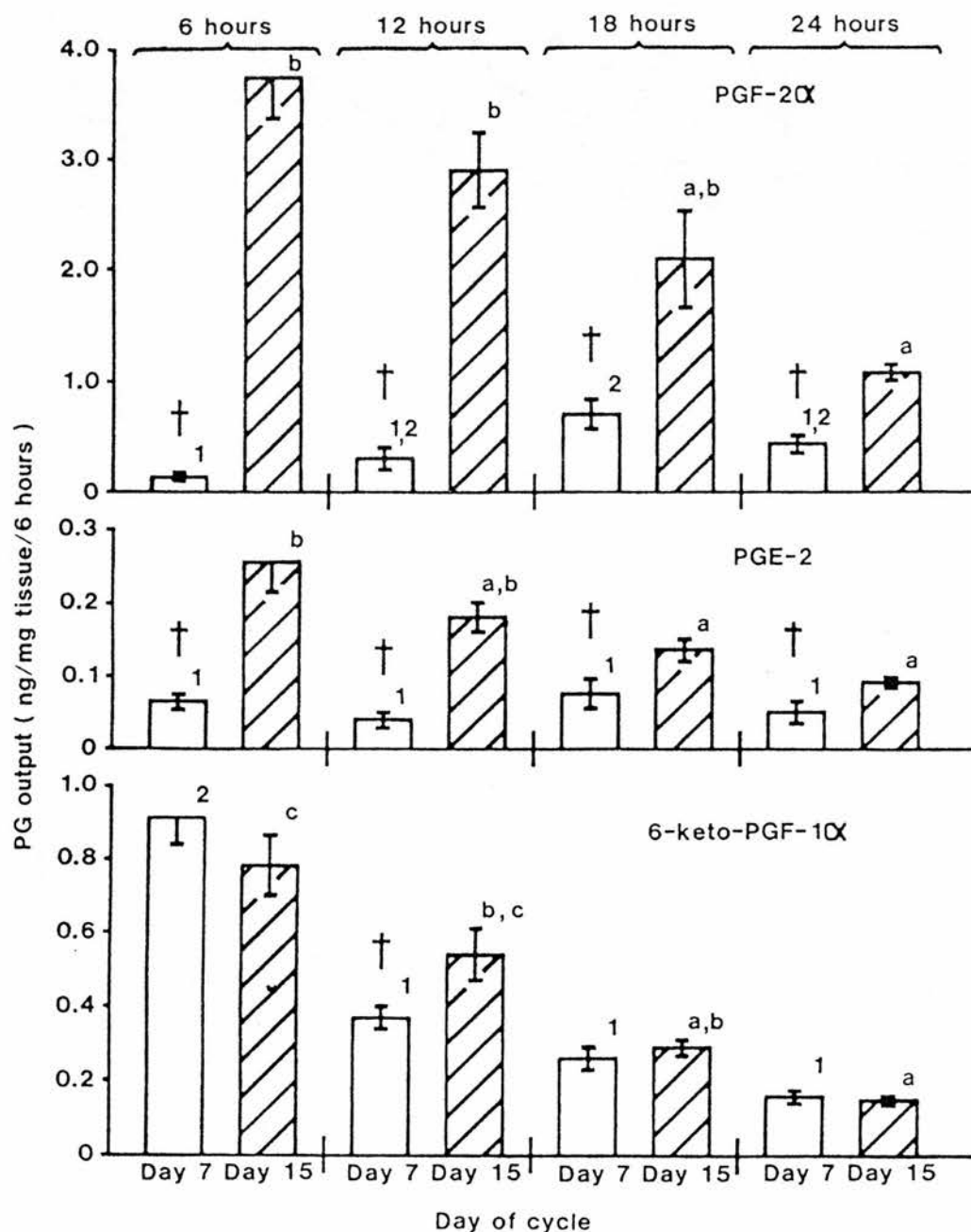


Figure 3.1.b

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from guinea-pig endometrium removed on day 7 (open columns) or on day 15 (hatched columns) of the oestrous cycle and cultured for 24h, with sampling every 6h.

† output significantly ( $P < 0.05$ ) lower compared to day 15 output for same PG during the same sample period.

superscripts: columns with the same superscript are not significantly ( $P < 0.05$ ) different for the same PG from tissue removed on the same day of the oestrous cycle.

cultured guinea-pig endometrium is in agreement with the increase in  $\text{PGF}_{2\alpha}$  concentrations in the utero-ovarian venous plasma of the guinea-pig at the end of the oestrous cycle (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975; Antonini, Turner and Pauerstein, 1976). A study on the superfused guinea-pig uterus by Poyser and Brydon (1983) showed that  $\text{PGF}_{2\alpha}$  output increased 21.9-fold between days 7 and 15 of the oestrous cycle, while  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs only increased 2.9- and 1.2-fold, respectively. The increases in PG outputs between day 7 and day 15 during the first 6h of culture were 26.5-fold for  $\text{PGF}_{2\alpha}$  and 4.5-fold for  $\text{PGE}_2$ , while the output of 6-keto- $\text{PGF}_{1\alpha}$  was slightly less from day 15 than from day 7 endometrium. During this first six hours of culture there appears to be a large degree of comparability in the PG outputs with the much shorter superfusion experiments.

The decline in PG output from the endometrium in culture for several days has been previously reported for human endometrium (Abel and Baird, 1980; Tsang and Ooi, 1982) and for guinea-pig endometrium cultured for three days (Leaver and Seawright, 1982). However, there was an increase in  $\text{PGF}_{2\alpha}$  output between 12h and 18h of culture from day 7 endometrium. This increase may have been due to removing the endometrium from the influence of progesterone that is present in vivo. Plasma progesterone concentrations are high at this stage of the cycle in the guinea-pig (Joshi, Watson and Labhsetwar, 1973) and also in the sheep (Ottobre, Lewis, Thayne and Inskeep, 1980). Vincent and Inskeep (1986) have shown that after ovariectomy of sheep on day 12 of the oestrous cycle,  $\text{PGF}_{2\alpha}$  concentrations in the utero-ovarian vein rise rapidly on day 13 but, if progesterone was replaced, the  $\text{PGF}_{2\alpha}$  concentrations were not different from intact ewes. Thus, progesterone has an inhibitory effect on endometrial  $\text{PGF}_{2\alpha}$  synthesis

in the sheep. A similar action in the guinea-pig could explain why removing the endometrium on day 7 from intact guinea-pigs and placing it in culture (away from the inhibitory action of progesterone) results in the increase in endometrial  $\text{PGF}_{2\alpha}$  output.

This experiment has shown the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig endometrium maintained in culture for three days, and also in more detail, the profile of the outputs of these PGs during the initial 24h period of culture. These outputs of PGs from untreated (control) endometrium maintained in culture can be compared with endometrium subjected to a variety of different treatments that are presented in the following results sections (sections 3.2, 3.3, 3.5 and 3.6).

### 3.2 THE EFFECT OF INDOMETHACIN ON PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG ENDOMETRIUM IN CULTURE

#### INTRODUCTION

Indomethacin is a non-steroidal anti-inflammatory drug that inhibits PG synthesis by inhibiting the cyclo-oxygenase component of the PGH synthase enzyme, which converts arachidonic acid to PGH<sub>2</sub> (Rome and Lands, 1975). Indomethacin has been shown to increase the length of the oestrous cycle in the guinea-pig (Marley, 1972; Horton and Poyser, 1973) an effect that was proposed to be caused by the inhibition of PGF<sub>2α</sub> synthesis by the guinea-pig uterus (Poyser and Horton, 1975). Subsequently, Poyser (1985a) showed that indomethacin inhibits basal PGF<sub>2α</sub> output from the superfused guinea-pig uterus in vitro when added to the superfusing solution.

This study has investigated PG synthesis in the guinea-pig endometrium by examining the effect of indomethacin on PG output from and PG synthesizing capacity of the guinea-pig endometrium in culture for up to 24h. As PGs are synthesised immediately before release and are not stored (Ramwell, Leovey and Sintetos, 1977), the main aim of this study was to show that PG synthesis occurred during tissue culture.

#### METHODS

Guinea-pigs on day 7 and day 15 of the oestrous cycle were killed, and their uteri were removed in preparation for tissue culture as described previously (section 2.2.1). Endometrium was dissected from each uterus and small pieces of endometrium were placed in 24 Petri dishes, each dish containing 12-20mg tissue (see section 2.2.5). The dishes were divided into six groups of four dishes and were treated as follows; two groups were treated with 2μg indomethacin/ml

(added in 10 $\mu$ l ethanol), two groups were treated with 10 $\mu$ g indomethacin/ml (added in 10 $\mu$ l ethanol) and the final two groups were treated with 10 $\mu$ l ethanol (controls). The dishes were distributed among three Kilner jars, so that in each jar there were two different groups of dishes of each treatment (i.e. 8 dishes/jar). The jars were gassed and then incubated for 24h, sampling every 6h, in the conditions specified in section 2.2.5.

Immediately after the jars were placed in the incubator, two amounts of the remaining endometrium, each weighing approximately 12-20mg, were accurately weighed, homogenised and incubated for 60 min with 2 $\mu$ g sodium arachidonate (dispensed in 10 $\mu$ l ethanol) according to the method and conditions described in section 2.2.4.ii. These two homogenates of endometrium measured the "0h" control PG synthesizing capacity of the tissue. After incubation, PGs were extracted from the incubates and redissolved in 5ml ethyl acetate as described in section 2.2.2.

After 6h, 12h and 18h of culture, one dish was removed from each group of dishes at each time. The medium from the remaining dishes was collected, replaced with fresh medium, and the same dose of indomethacin or ethanol was added. These dishes were then replaced in the jars, which were regassed and returned to the incubator in the conditions previously specified. After 24h of culture, the remaining six dishes were removed.

The culture medium from the removed dishes was collected. The tissue was then weighed, homogenised and incubated with 2 $\mu$ g sodium arachidonate. After 60 min incubation, the PGs were extracted, as described for "0h" controls above. These homogenates measured the endometrial PG synthesizing capacities after 6h, 12h, 18h and 24h of culture. All samples of a culture medium and ethyl acetate extracts

of incubate samples were stored at  $-20^{\circ}\text{C}$  before the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present were measured by radioimmunoassay (see section 2.3).

#### Statistical tests

PG outputs from or synthesizing capacities of treated tissues were compared with their respective control values for the same PG at the same time using Student's 't' test. Duncan's multiple-range test was used to compare PG production by "0h" control endometrial homogenates with PG production by control or treated endometrial homogenates, after different periods in culture; this test was also used to analyse changes in the outputs of PGs released into the culture medium with time.

#### RESULTS

The output of  $\text{PGF}_{2\alpha}$  from control day 7 guinea-pig endometrium was greatest during the first 6h of culture (Figure 3.2.a). The output of  $\text{PGF}_{2\alpha}$  declined significantly ( $P < 0.05$ ) between 6h and 12h, then increased between 12h and 18h, and this level was maintained up to 24h (Figure 3.2.a). Control outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 endometrium declined significantly ( $P < 0.05$ ) during the 24h period of culture, from an initial highest output during the first 6h of culture (Figure 3.2.a).

Indomethacin treatment of day 7 guinea-pig endometrium at both concentrations (2 and  $10\mu\text{g/ml}$ ) significantly ( $P < 0.05$ ) inhibited the increase in  $\text{PGF}_{2\alpha}$  output exhibited by control tissues between 12h and 18h, and this inhibition was maintained for the remainder of the culture period (Figure 3.2.a). Both 2 and  $10\mu\text{g/ml}$  concentrations of indomethacin significantly ( $P < 0.05$ ) inhibited  $\text{PGE}_2$  output from day 7 endometrium during the first 6h of culture and significantly ( $P <$

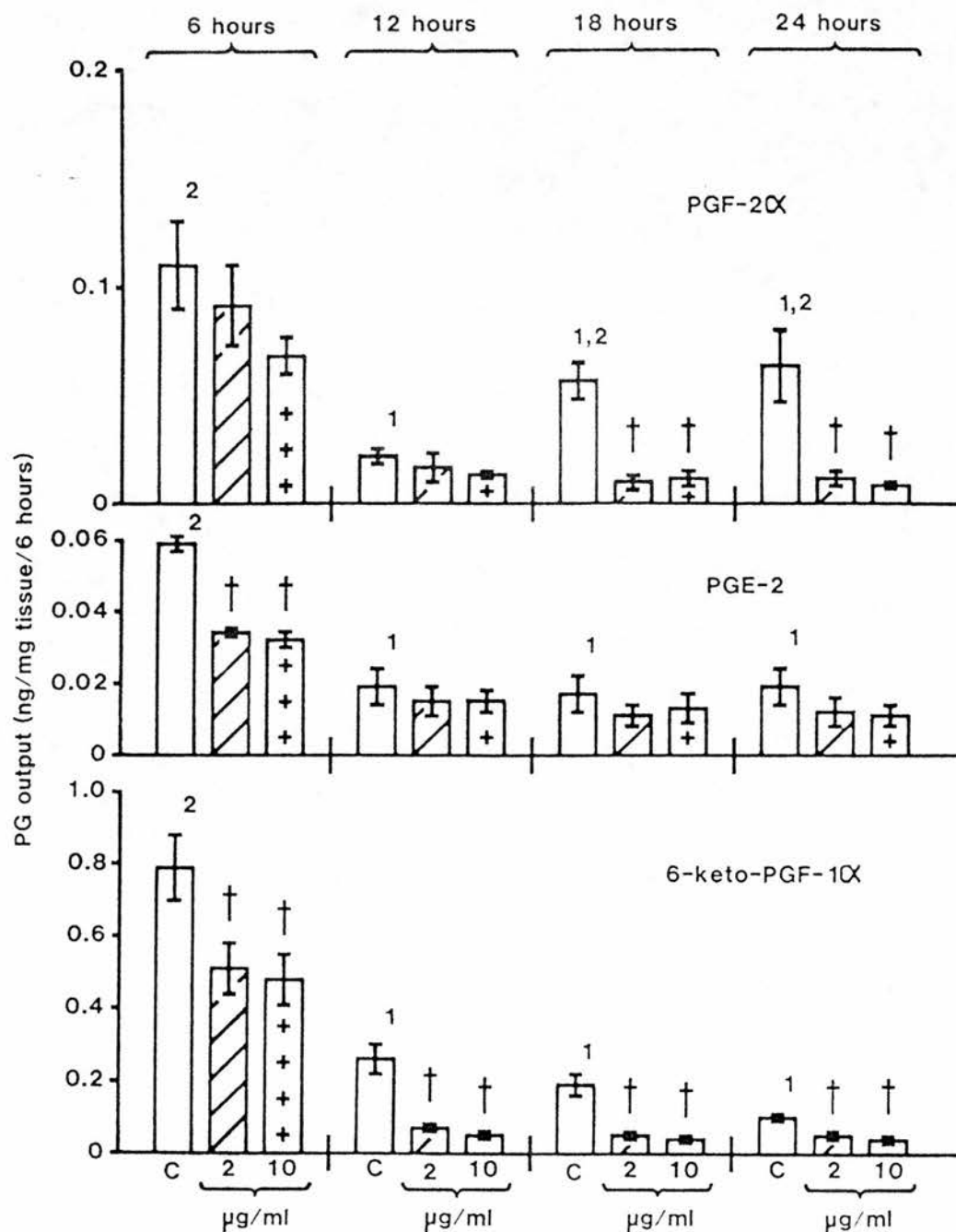


Figure 3.2.a

Mean ( $\pm$  s.e.m.;  $n = 6$ ) PG outputs (ng/mg tissue/6h) from guinea-pig endometrium removed on day 7 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h in the absence (controls (C); open columns) or presence of 2μg/ml (hatched columns) or 10μg/ml indomethacin (crossed columns).

† significantly ( $P < 0.05$ ) lower than control (C) PG output at the same time for the same PG.

Different superscripts on control (C) columns for the same PG indicate they are significantly ( $P < 0.05$ ) different.



0.05) inhibited 6-keto-PGF<sub>1α</sub> output from day 7 endometrium throughout the 24h period of culture, when compared to control outputs at the same time (Figure 3.2.a).

The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from control endometrium obtained from day 15 guinea-pigs were significantly ( $P < 0.05$ ) higher during the first 6h of culture than during the next three culture periods (Figure 3.2.b). The outputs of all PGs declined steadily over the 24h period of culture. Indomethacin at both concentrations of 2 and 10μg/ml significantly ( $P < 0.05$ ) inhibited the outputs of all three PGs during every 6h sample period throughout the 24h culture, when compared to controls at the same time for the same PG (Figure 3.2.b).

The amounts of PGF<sub>2α</sub> synthesised by control day 7 guinea-pig endometrium homogenised after culture increased steadily with time and, at 24h, production was significantly ( $P < 0.05$ ) higher than at 0h (Figure 3.2.c). The amounts of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesised by day 7 endometrium homogenised after culture did not significantly change with time (Figure 3.2.c). Indomethacin treatment at both concentrations of 2 and 10μg/ml significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> production by day 7 endometrium homogenised after 6h, 12h, 18h and 24h of culture, when compared both to their respective 0h control values and to their respective control values at the same time, except 10μg indomethacin/ml treatment did not significantly inhibit the PGE<sub>2</sub> synthesising capacity after 6h, 12h and 18h to a value lower than the 0h control value (Figure 3.2.c).

Cultured, control endometrium from day-15 guinea-pigs after homogenisation and incubation showed no significant change in the amounts of any PG produced at any time when compared to 0h control homogenate values, for the same PG (Figure 3.2.d). Indomethacin at

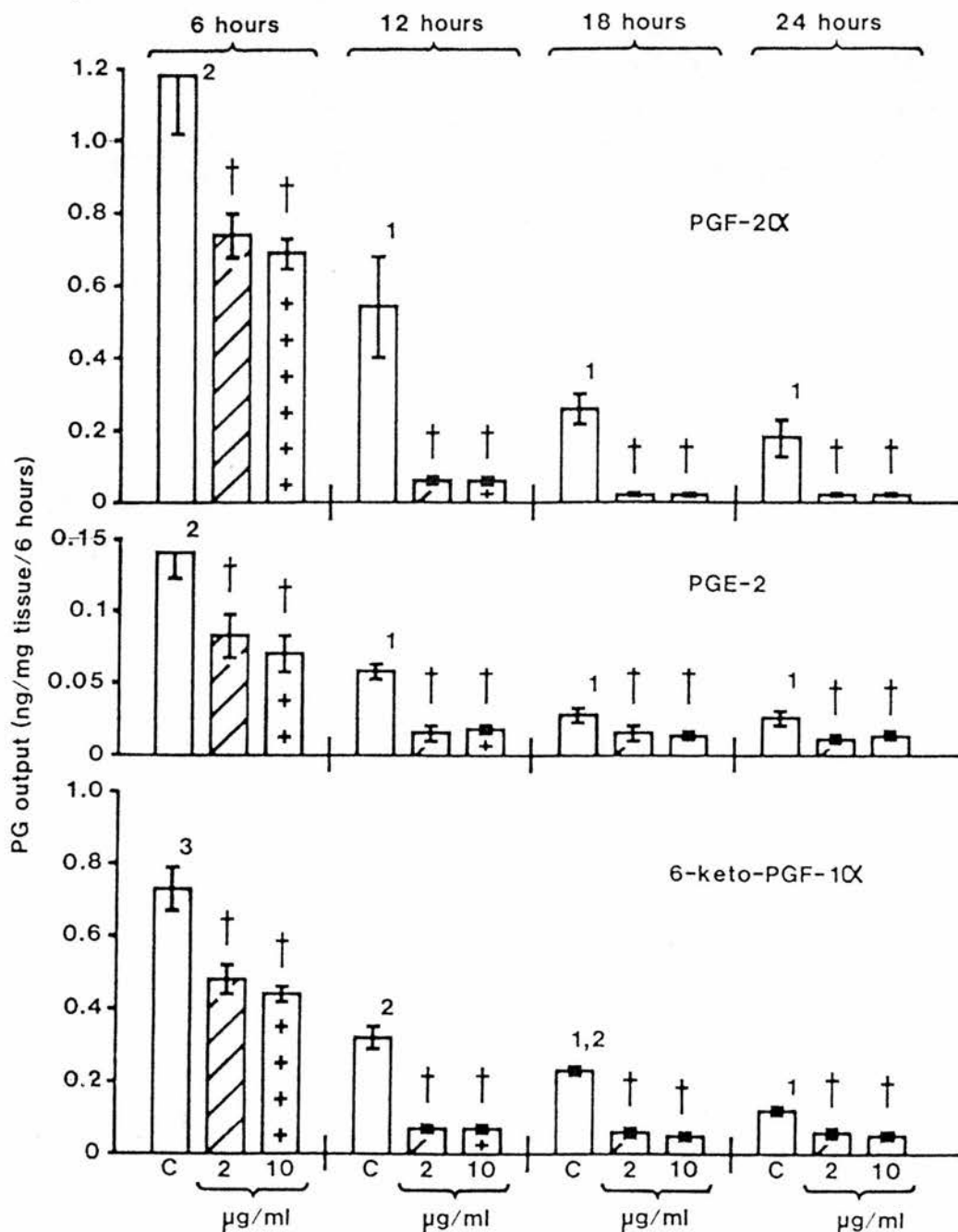


Figure 3.2.b

Mean ( $\pm$  s.e.m.;  $n = 6$ ) PG outputs (ng/mg tissue/6h) from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h in the absence (controls (C); open columns) or presence of 2 $\mu$ g/ml (hatched columns) or 10 $\mu$ g/ml indomethacin (crossed columns).

† significantly ( $P < 0.05$ ) lower than control (C) PG output at the same time for the same PG.

Different superscripts on control (C) columns for the same PG indicate they are significantly ( $P < 0.05$ ) different.

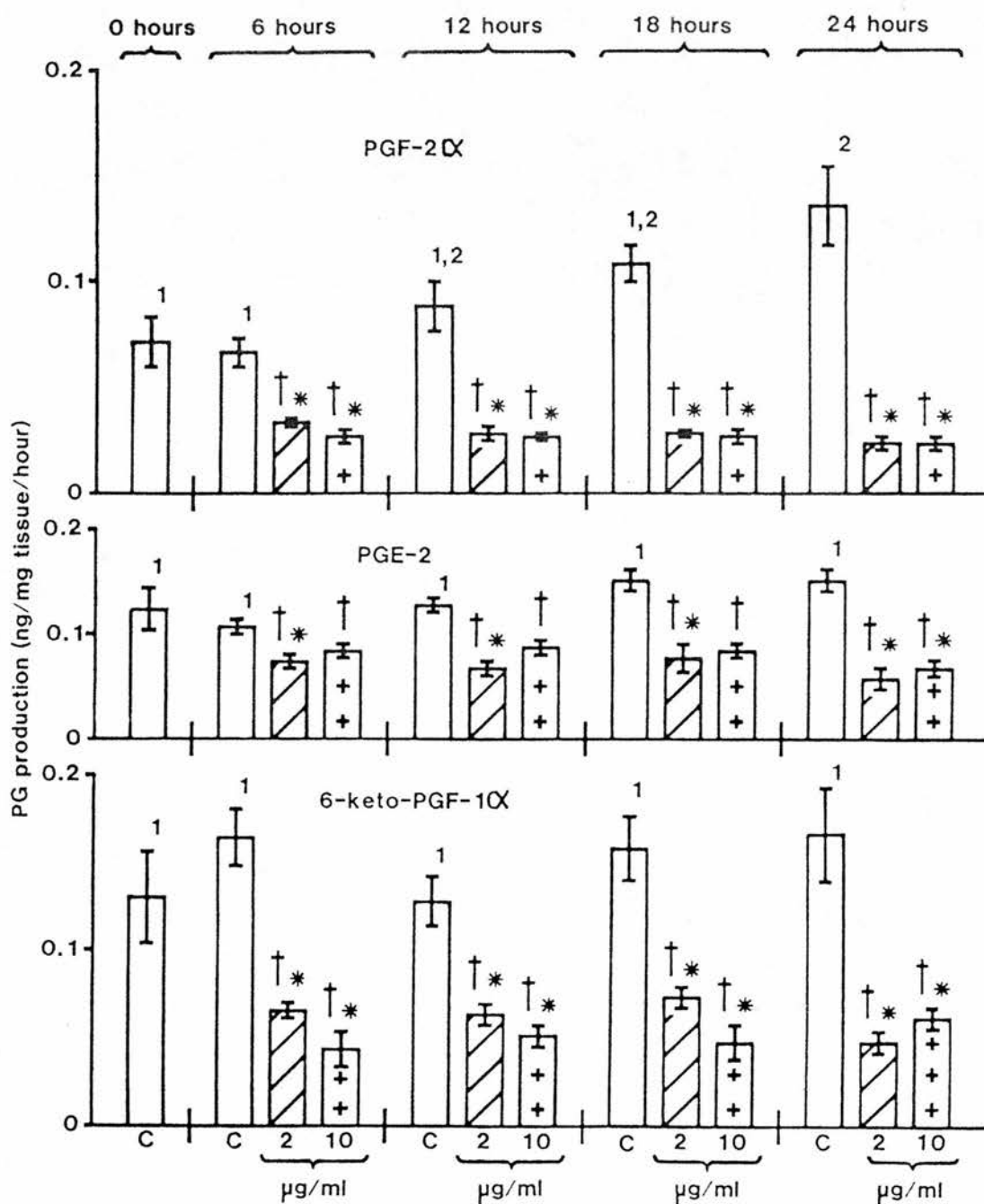


Figure 3.2.c

Mean ( $\pm$  s.e.m.;  $n = 6$ ) production of PGs (ng/mg tissue/h) synthesised by homogenates of day 7 guinea-pig endometrium either without culture and without treatment (control (C); open columns) at 0h or after 6h, 12h, 18h and 24h of culture in the absence (control (C); open columns) and in the presence of 2 $\mu$ g/ml (hatched columns) or 10 $\mu$ g/ml indomethacin (crossed columns).

† significantly ( $P < 0.05$ ) lower than control PG production at the same time, for the same PG.

\* significantly ( $P < 0.05$ ) lower than control PG production at 0h for the same PG.

Different superscripts on control (C) columns for the same PG indicate they are significantly ( $P < 0.05$ ) different.

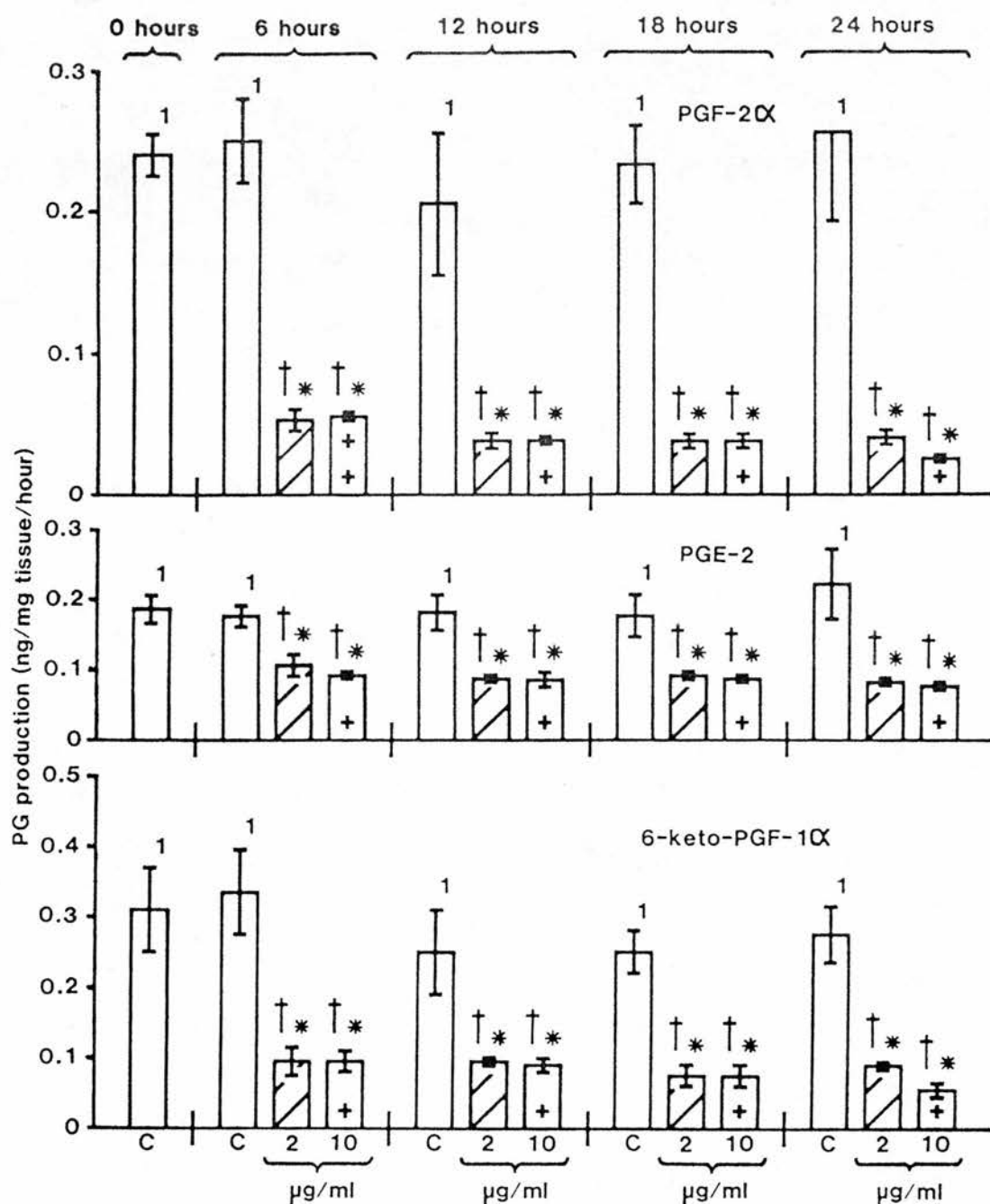


Figure 3.2.d

Mean ( $\pm$  s.e.m.;  $n = 6$ ) production of PGs (ng/mg tissue/h) synthesised by homogenates of day 15 guinea-pig endometrium either without culture and without treatment (control (C); open columns) at 0h, or after 6h, 12h, 18h and 24h of culture in the absence (control (C); open columns) and in the presence of 2 $\mu$ g/ml (hatched columns) or 10 $\mu$ g/ml indomethacin (crossed columns).

† significantly ( $P < 0.05$ ) lower than control PG production at the same time for the same PG.

\* significantly ( $P < 0.05$ ) lower than control PG production at 0h for the same PG.

Different superscripts on control (C) columns for the same PG indicate they are significantly ( $P < 0.05$ ) different.

both concentrations (2 and 10 $\mu$ g/ml) significantly ( $P < 0.05$ ) inhibited PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  production by day 15 endometrium homogenised after 6h, 12h, 18h and 24h of culture, when compared both to their respective control values measured at the same time and to their respective 0h control values (Figure 3.2.d).

The amounts of PGF<sub>2 $\alpha$</sub>  and 6-keto-PGF<sub>1 $\alpha$</sub>  produced by control endometrial homogenates were significantly ( $P < 0.05$ ) higher on day 15 than on day 7 at all sample times (Figure 3.2.c and 3.2.d). The amounts of PGE<sub>2</sub> produced by day 15 control endometrial homogenates at every sample time were not significantly different from day 7 endometrial homogenates. Similar amounts of 6-keto-PGF<sub>1 $\alpha$</sub>  and PGE<sub>2</sub>, with slightly less PGF<sub>2 $\alpha$</sub> , were synthesized by homogenates of day 7 tissue (Figures 3.2.c and 3.2.d).

## CONCLUSIONS

The basal outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  from day 7 and day 15 guinea-pig endometrium in culture for up to 24h presented here are similar to the PG outputs and profiles of PG outputs presented in section 3.1 (see Figure 3.1.b).

Treatment with indomethacin prevented the increase in PGF<sub>2 $\alpha$</sub>  output from day 7 endometrium which occurs after 12h in culture, and also inhibited PGF<sub>2 $\alpha$</sub>  output from day 15 endometrium throughout the culture period. Indomethacin treatment inhibited PGE<sub>2</sub> output from both day 7 and day 15 cultured endometrium throughout the 24h culture period, although this effect on output was not significant between 6h and 24h for day 7 endometrium due to the very low output of PGE<sub>2</sub>. Indomethacin treatment inhibited 6-keto-PGF<sub>1 $\alpha$</sub>  output from both day 7 and day 15 guinea-pig endometrium throughout the 24h culture period. Indomethacin appeared to exert a non-specific inhibitory action on all

PG outputs from guinea-pig endometrium in culture for 24h, which is in agreement with indomethacin inhibiting the cyclo-oxygenase component of PGH synthase.

Indomethacin significantly inhibited PG synthesis by homogenates of day 7 and day 15 endometrium at the end of each culture period (i.e. the PG synthesizing capacity; this term refers to PG production by homogenised and therefore broken cell preparations). This inhibitory effect of indomethacin treatment on PG synthesizing capacity appears to be an irreversible blockade (or a very slow reversible blockade), because no further indomethacin was added to the incubating medium used for the homogenates, and therefore the block was not reversed by this dilution of the tissue and cell contents after homogenization.

## DISCUSSION

It can be seen from these results that there is little correlation between the basal PG outputs and the PG synthesizing capacities for cultured endometrium when making a comparison between the same and also different days of the oestrous cycle. The output of  $\text{PGF}_{2\alpha}$  from the first 6h of culture from day 15 endometrium was 10.8-fold higher than from day 7 endometrium, although the synthesizing capacity was only 3.8-fold higher. The  $\text{PGF}_{2\alpha}$  synthesizing capacity did not increase to the same extent as  $\text{PGF}_{2\alpha}$  output between day 7 and day 15. Also, the output of  $\text{PGF}_{2\alpha}$  from day-15 endometrium significantly declined by up to 80% during the 24h period of culture, but there was no significant change in the  $\text{PGF}_{2\alpha}$  synthesizing capacity throughout the 24h culture period. This decrease in PG output without a change in the synthesizing capacity is seen elsewhere;  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from both day 7 and day

15 tissue significantly decreased from a maximal output during the first 6h of culture, although there was no significant change in their synthesizing capacities. From this evidence, PG output from either day 7 or day 15 guinea-pig endometrium in culture does not appear to be controlled by their PG synthesizing capacity.

Prostaglandin H synthase has two enzymic activities; a cyclo-oxygenase activity that converts arachidonic acid into the hydroperoxide, PGG<sub>2</sub>, and a peroxide activity that reduces PGG<sub>2</sub> to PGH<sub>2</sub> (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976). Subsequent steps in PG biosynthesis lead very rapidly (1-2 min) to the formation of PGs and thromboxanes including PGF<sub>2α</sub>, PGE<sub>2</sub> and PGI<sub>2</sub> from PGH<sub>2</sub> (Hamberg, Svensson, Wakabayashi and Samuelsson, 1974). Indomethacin is a selective inhibitor of the cyclo-oxygenase step and this effect was found to be almost irreversible (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976; Mizumo, Yamamoto and Lands, 1982). Non-steroidal drugs have been shown to have different potencies at inhibiting PG synthesis in different tissues (Shen, 1977). Hahn, Carraher and McGuire (1982) have shown that indomethacin is a highly potent inhibitor of PGF<sub>2α</sub>-induced contractions of the guinea-pig uterus. Interestingly, Tolman, Fuller and Rosenthale (1985) demonstrated that the non-steroidal anti-inflammatory drug, suprofen was a more potent inhibitor of PGF<sub>2α</sub> and PGE<sub>2</sub> production than of 6-keto-PGF<sub>1α</sub> production by guinea-pig uterine homogenate preparations. Unlike suprofen, indomethacin treatment of guinea-pig endometrium was non-selective in its PG inhibitory action.

Indomethacin administered by a slow releasing preparation placed in the uterine lumen of the guinea-pig has been shown to increase the oestrous cycle length (Marley, 1972; Poyser and Horton, 1973). However, oral or parenteral administration of indomethacin has little



effect on increasing oestrous cycle length (cycle length was only increased by up to 3 days) whereas local intra-uterine administration of indomethacin prolonged oestrous cycle length, in some cases to over 50 days, a similar effect to that produced by hysterectomy (Marley, 1973; Poyser and Horton, 1973). Subsequently, Poyser and Horton (1975) showed that in the guinea-pig an intra-uterine preparation of indomethacin lengthened the oestrous cycle (in one animal to 72 days) and plasma progesterone concentrations were higher throughout this extended cycle, indicating that functional corpora lutea were maintained. Indomethacin treatment has also been shown to prolong luteal function in the rabbit (O'Grady, Caldwell, Auletta and Speroff, 1972) and the rat (Chatterjee, 1973). Treatment with indomethacin ( $5.6\mu\text{M}$ ) of the day 15 guinea-pig uterus superfused in vitro inhibited the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (Poyser, 1985a). The outputs of all PGs from guinea-pig uterus were inhibited by indomethacin after a short delay (10-20 mins), and the inhibition produced by indomethacin appeared to be non-specific because the outputs of all three PGs were inhibited at a similar rate and to a similar extent (Poyser, 1985a). The results presented here are compatible with the previous studies that indomethacin inhibits the outputs of all PGs from the guinea-pig endometrium to a similar extent.

Concentrations of PGH synthase may reach nM concentrations in vivo. For instance, levels of  $10^4$  molecules of PGH synthase per platelet have been measured (Roth and Machuga, 1982). With a platelet count of  $2.5 \times 10^5$  per  $\text{mm}^3$ , human blood PGH synthase concentration may reach 4.2nM, and if all of this enzyme were activated at once, a theoretical pulse of  $0.7\mu\text{M}$   $\text{PGG}_2$  would result (Kulmacz, 1987). However, the amount of substrate and the amount of



initiator (i.e. fatty acid peroxides) for the cyclo-oxygenase are the limiting factors for PG synthesis in vivo, and therefore only a small amount of the total PGH synthase enzyme available would be synthesizing PGs at any one time (Marshall, Kulmacz and Lands, 1987).

The measurement of PG synthesis by broken cell homogenates (termed synthesizing capacity here) is not a direct measure of the potential for PG production by the intact tissue because, as stated above, the PGH synthase released by homogenisation in vitro is not all activated at the same time in vivo. Concentrations of free arachidonic acid released after homogenisation of the guinea-pig uterus but before incubation of the homogenates are high (2 $\mu$ g free arachidonic acid/100mg tissue; Mitchell, Poyser and Wilson, 1977). Furthermore, after incubation for 60 min, concentrations of free arachidonic acid increased further during the incubation period to about 5 $\mu$ g free arachidonic acid/100mg tissue. Mitchell, Poyser and Wilson (1977) also showed there was no difference in the amounts of free arachidonic acid released by homogenates of day 7 and day 15 guinea-pig uterus, both before and after incubation. However, more prostaglandins were produced by day 15 than by day 7 uterine homogenates. It was calculated that about 1% of the available arachidonic acid from day 7 uterine tissue, and about 2-3% of the available arachidonic acid from day 15 tissue was converted to prostaglandins after incubation of homogenates of these tissues (Mitchell, Poyser and Wilson, 1977). Dimov, Christensen and Green (1983) also demonstrated that homogenisation of a variety of human reproductive tissues caused the release of substantial quantities ( $\mu$ g quantities of arachidonic acid released/g tissue) into the medium during incubation. Furthermore, in the present study exogenous sodium arachidonate was added to the incubates of homogenates to

ensure sufficient arachidonic acid substrate was available for conversion by PGH synthase to PGH<sub>2</sub>, and then to the measured PGs. Clearly, the limitation of prostaglandin synthesis in homogenates of guinea-pig uterus is not due to a lack of precursor, as occurs in vivo.

The activity of PGH synthase exhibits self-catalysed inactivation (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976; Ohki, Ogino, Yamamoto and Hayaishi, 1979). This means that as arachidonic acid is converted by PGH synthase to PGH<sub>2</sub>, the enzyme is inactivated and eventually (in less than 60 min) the reaction stops due to the lack of active enzyme rather than lack of substrate (Lands, LeTellier, Rome and Vanderhoek, 1972). Therefore, the amount of PGH synthase present determines the amount of PGH<sub>2</sub> and hence the amount of measured PGs formed. Tissue levels of PGs in guinea-pig endometrium have been determined by Poyser (unpublished results). After removal from the guinea-pig the endometrium was placed immediately in ethanol, which prevents PG production (Jouvenaz, Nugteren, Beerthius and Van Dorp, 1970; Green, 1979), and the tissue was homogenised. The PGs were extracted from the homogenates and were measured by radioimmunoassay. Tissue levels (all expressed as ng PG/mg tissue) of PGF<sub>2</sub> $\alpha$  in day 7 and day 15 tissue were  $0.024 \pm 0.005$  and  $0.81 \pm 0.15$ , respectively; the levels of PGE<sub>2</sub> were  $0.058 \pm 0.011$  and  $0.055 \pm 0.008$ , respectively; and the levels of 6-keto-PGF<sub>1</sub> $\alpha$  were  $0.038 \pm 0.006$  and  $0.043 \pm 0.006$ , respectively. The tissue levels of all the PGs were very low in both day 7 and day 15 endometrium. Therefore, the amounts of PGs present in incubates of homogenates produce a direct measure of the amount of PGH synthase present in the incubates available for PG synthesis. However, in the intact tissue, the supply of substrate in the rate-limiting step in PG synthesis by and release from the guinea-pig

endometrium. Since indomethacin treatment reduced the amounts of PGs present in the medium following culture, PG synthesis must have been occurring in the endometrium during culture.

### 3.3 CALCIUM AND ITS ROLE IN PROSTAGLANDIN PRODUCTION BY THE GUINEA- PIG ENDOMETRIUM

#### INTRODUCTION

Prostaglandin  $F_{2\alpha}$  output from the superfused guinea-pig uterus removed on day 15 of the oestrous cycle is stimulated by the calcium ionophore A23187 (Poyser and Brydon, 1983). This stimulation was abolished by using calcium-free superfusing medium (Poyser, 1984a), indicating that the response to A23187 is mediated by extracellular  $Ca^{2+}$  entering the cell. A23187 has only a weak stimulatory effect on  $PGE_2$  output. Phospholipase (PL)  $A_2$  also stimulates  $PGF_{2\alpha}$  and, to a much smaller extent,  $PGE_2$  output from guinea-pig uterus when superfused in vitro. Since the profile of PG output stimulated by A23187 and  $PLA_2$  are similar, these results suggest that the increase in PG synthesis activated by A23187 is due to an increase in intracellular calcium, and the consequent stimulation of endogenous  $PLA_2$  (Poyser, 1987a).  $PLA_2$  is a calcium-requiring enzyme, and it has been proposed that oestradiol acts upon a progesterone-primed uterus to produce a rise in the endometrial intracellular calcium concentration, which stimulates  $PLA_2$  to release arachidonic acid from phospholipids for  $PGF_{2\alpha}$  production at the end of the cycle (Downing and Poyser, 1983).

This study has further investigated the involvement of calcium in PG synthesis in the guinea-pig uterus, by measuring PG outputs from guinea-pig endometrium in tissue culture by addressing the following questions:-

- (a) How much does the high output of  $PGF_{2\alpha}$  from endometrium removed on day 15 of the oestrous cycle depend on intracellular and extracellular calcium ?
- (b) If extracellular calcium is involved, does the calcium enter the

cell by voltage-dependent calcium channels ?

(c) Does calmodulin, a calcium-binding protein (Cheung, 1980) that has been implicated in the stimulation of PLA<sub>2</sub> (Moskowitz, Andres, Silva, Shapiro, Schook and Puszkin, 1985), have a role in increasing PGF<sub>2α</sub> output from day 15 endometrium ?

(d) Are the mechanisms controlling PGF<sub>2α</sub> synthesis also controlling PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesis ?

(e) Lipocortin inhibits the activity of PLA<sub>2</sub> (Blackwell and Flower, 1983) and lipocortin has been demonstrated to be released from human endometrium in culture (Gurpide, Markiewicz, Schatz and Hirata, 1986). In platelets, a lipocortin-like protein is inactivated by phosphorylation by the enzyme protein kinase C (Touqui, Rothhurt, Shaw, Fradin, Vargaftig, and Russo-Marie, 1986). Is protein kinase C and hence a lipocortin-like protein involved in the control of PG synthesis in guinea-pig endometrium ?

## METHODS

Guinea-pigs were killed and their uteri removed in preparation for tissue culture (section 2.2.1). Endometrium was dissected away from the myometrium and small pieces were placed in Petri dishes for tissue culture according to the method described in section 2.2.5. Each Petri dish contained 10-15mg tissue. Eighteen Petri dishes were prepared from the endometrium of each uterus. Four of these dishes were left untreated (controls), the remaining fourteen dishes contained the treatments administered in duplicate, as specified in the individual experimental methods below. These dishes were then divided equally, with nine dishes being placed into two modified Kilner jars. Each group of nine dishes contained two control dishes and one dish from each of the duplicate treatments. The endometrium

was cultured for 72h in the conditions specified in section 2.2.5. The culture medium was changed every 24h, and the dishes were replaced in the jars, regassed and incubated as above. The samples of culture medium were stored at -20°C before the amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> present were measured by radioimmunoassay (see section 2.3).

1. Experimental treatments using Ca<sup>2+</sup>-containing medium

Endometrium was used from guinea-pigs killed on day 15 of the oestrous cycle (day 15 endometrium). All eighteen dishes from each uterus contained Ca<sup>2+</sup>-containing Medium 199 with supplements as described in section 2.2.5. The following compounds were utilised, in duplicate for each treatment: ethyleneglycol-bis-(β-aminoethyl ether acid (EGTA; a calcium chelator; 2mM), 8-(N,N'-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8; an intracellular Ca<sup>2+</sup> antagonist; 30 and 100μM; Chiou and Malagodi, 1975), trifluoperazine (TFP; a calmodulin antagonist; 100 and 200μM; Levin and Weiss, 1977), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7; a calmodulin antagonist; 150 and 300μM; Hidaka, Asano, Iwadare, Mutsumoto, Totsuka and Aoki, 1978), nifedipine (1, 10 and 100μM; dispensed in 20μl ethanol) and verapamil (1, 10, 50 and 100μM; dispensed in 20μl saline) (both voltage-dependent Ca<sup>2+</sup>-channel blockers; Janis and Scriabine, 1985) and phorbol 12-myristate 13-acetate (TPA; a stimulator of protein kinase C; 1, 5 and 25ng/ml dispensed in 20μl ethanol; Castagna, Takai, Kaibuchi, Sano, Kikkawa and Nishizuka, 1982).

Also, endometrium removed from guinea-pigs on day 7 of the oestrous cycle (day 7 endometrium) was cultured in four untreated day 7 control dishes and in six dishes containing one of the duplicate concentrations of TPA used (1, 5, 25ng/ml). The dishes were divided

equally as before between two Kilner jars, each containing two day 7 control dishes and one of each duplicate treatment. The dishes were incubated for 72h, with sampling every 24h, using the same procedure as for day 15 tissue.

All experiments that required the preparation and dispensing of nifedipine and verapamil were performed under light from a sodium lamp due to the instability of these compounds in UV light.

## 2. Experimental treatments using $\text{Ca}^{2+}$ -depleted medium

Day 15 guinea-pig endometrium was prepared for tissue culture in eighteen dishes as before. Four of these dishes were prepared containing  $\text{Ca}^{2+}$ -containing medium ( $\text{Ca}^{2+}$ -containing medium controls), while the remaining dishes were prepared containing  $\text{Ca}^{2+}$ -depleted medium. Four of these remaining fourteen dishes received no further treatment ( $\text{Ca}^{2+}$ -depleted medium controls). The remaining ten dishes were treated, in duplicate, with EGTA (2mM), TMB-8 (100 $\mu\text{M}$ ), W-7 (150 $\mu\text{M}$ ), nifedipine (100 $\mu\text{M}$ ) and verapamil (100 $\mu\text{M}$ ). The dishes were divided equally between two Kilner jars as before, each jar containing two of each of the two groups of controls (i.e.  $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -depleted medium controls) and one of each of the duplicate treatments. The jars were incubated for 72h, and sampled every 24h, using the protocol specified above.

## Presentation of results and statistical tests

From one uterus, there was insufficient endometrial tissue to prepare sufficient petri dishes to allow the different compounds at all the concentrations used to be compared with one set of control dishes. Therefore, to allow comparison between different sets of dishes using different controls, data are expressed as a percentage of the day 1 control value of that set of dishes for the same PG. The control value was obtained by taking the average of the two control

values for each Kilner jar. Treatment with different concentrations of a compound were performed in the same set of dishes using the same control values.

Results were analysed using the one- or two-tailed Student's 't' test as appropriate. The outputs from treated endometrium in  $\text{Ca}^{2+}$ -containing medium were compared to their respective control outputs for the same PG during the same time period. The outputs of PGs from treated endometrium cultured in  $\text{Ca}^{2+}$ -depleted medium were compared to their respective  $\text{Ca}^{2+}$ -depleted control output values for the same PG during the same time period.

## RESULTS

### 1. Using $\text{Ca}^{2+}$ -containing medium

Basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium cultured in  $\text{Ca}^{2+}$ -containing medium for three days have already been described in section 3.1.

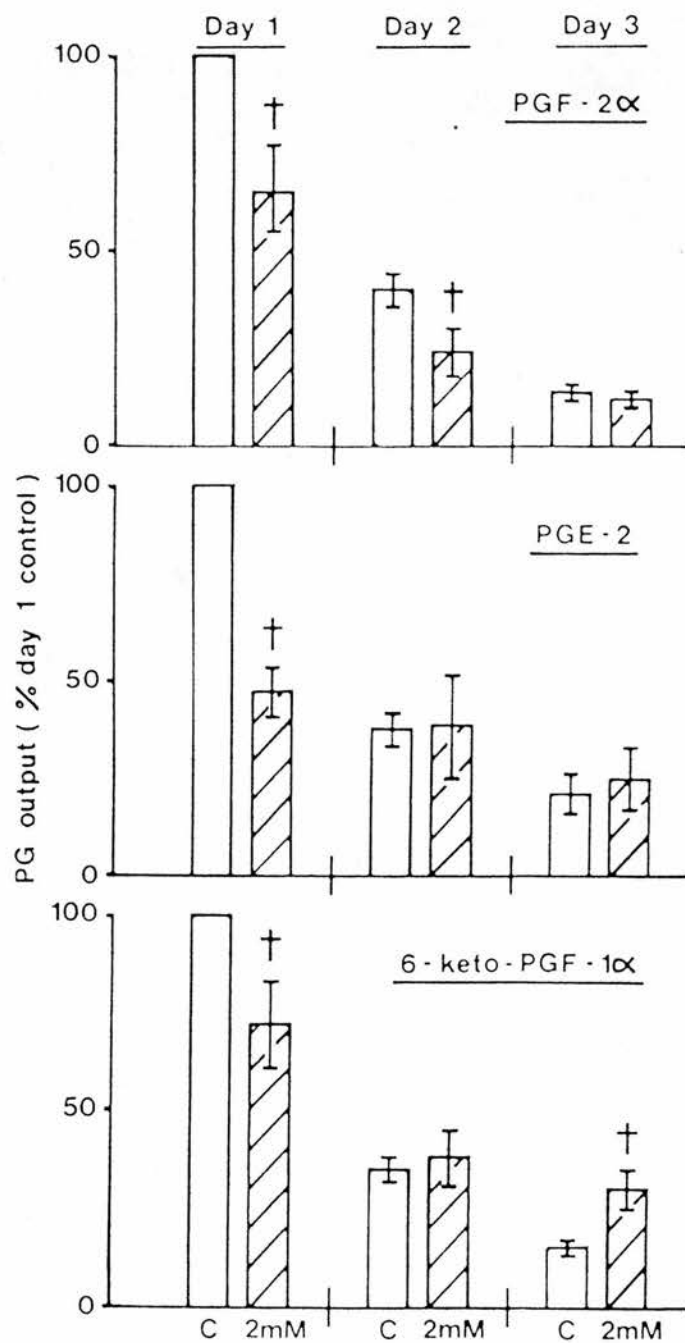
#### Effects of various treatments on PG output

The effects of EGTA, TMB-8, TFP, W-7, nifedipine and verapamil on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 guinea-pig endometrium in culture are shown in Figures 3.3.a, 3.3.b, 3.3.c, 3.3.d, 3.3.e and 3.3.f, respectively.

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were significantly ( $P < 0.05$ ) reduced on day 1 of culture by EGTA (2mM) (Figure 3.3.a). EGTA also significantly ( $P < 0.05$ ) reduced  $\text{PGF}_{2\alpha}$  output on day 2 of culture and significantly ( $P < 0.05$ ) increased 6-keto- $\text{PGF}_{1\alpha}$  output on day 3 of culture (Figure 3.3.a).

The output of  $\text{PGF}_{2\alpha}$  was significantly ( $P < 0.05$ ) inhibited on all three days of culture by both concentrations (30 and  $100\mu\text{M}$ ) of TMB-8 (Figure 3.3.b).  $\text{PGE}_2$  output was significantly ( $P < 0.05$ )





**Figure 3.3.a**

Mean (± s.e.m.; n = 10) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca<sup>2+</sup>-containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence (hatched columns) of 2mM EGTA.

† significantly (P < 0.05) different compared to the control value for the same PG on the same day of culture.

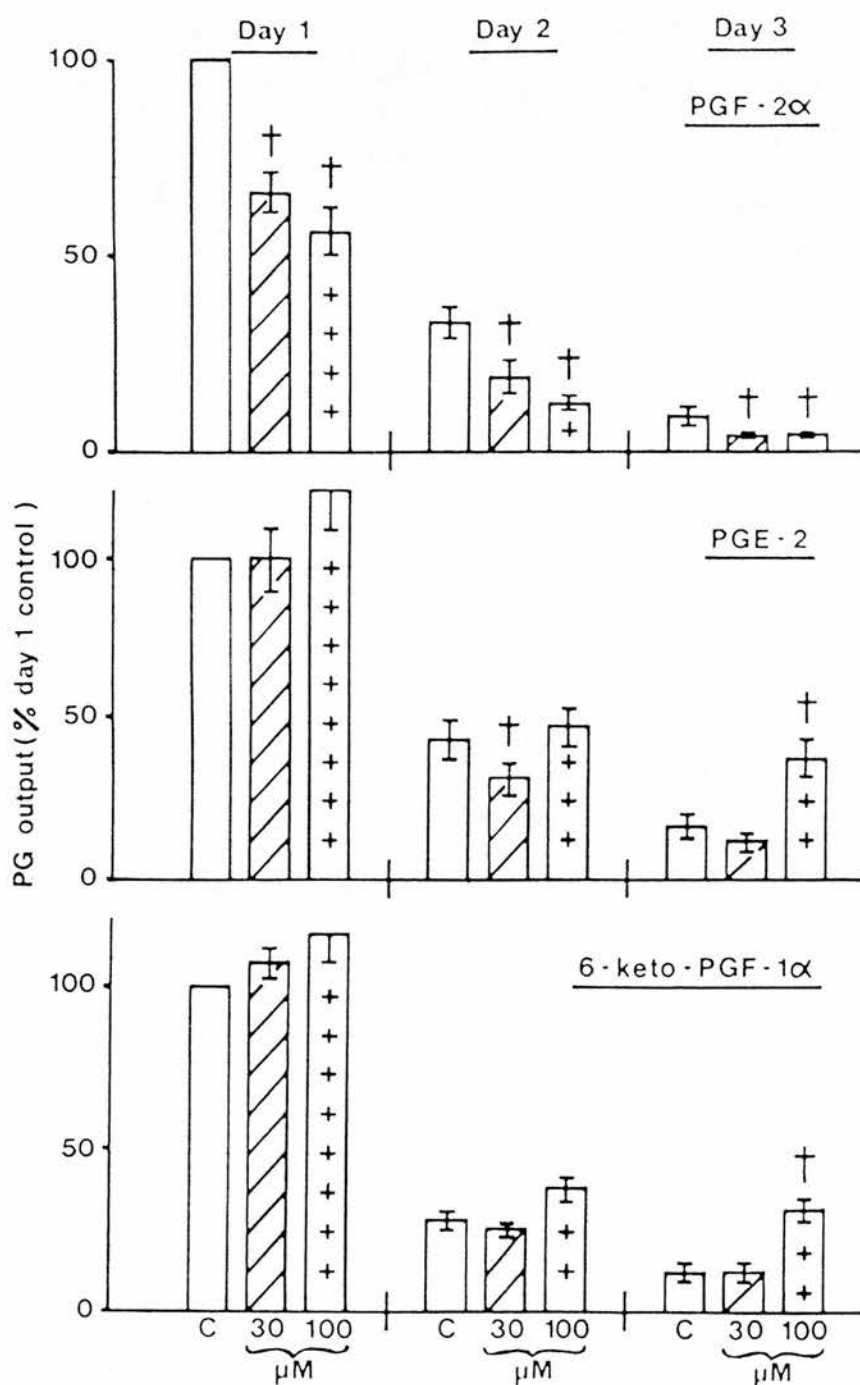


Figure 3.3.b

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in  $\text{Ca}^{2+}$ -containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of  $30\mu\text{M}$  (hatched columns) and  $100\mu\text{M}$  (crossed columns) TMB-8.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

reduced by 30 $\mu$ M TMB-8 on day 2 of culture, and 100 $\mu$ M TMB-8 significantly ( $P < 0.05$ ) increased the outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  on day 3 of culture (Figure 3.3.b).

Trifluoperazine (TFP; 200 $\mu$ M) significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output on day 1 of culture, 100 $\mu$ M TFP significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output on day 2 of culture, and TFP (100 and 200 $\mu$ M) significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output on day 3 of culture (Figure 3.3.c). The outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  were significantly ( $P < 0.05$ ) increased by TFP (100 and 200 $\mu$ M) on all three days of culture, with the exception of 100 $\mu$ M TFP treatment on 6-keto-PGF<sub>1 $\alpha$</sub>  output on day 3 of culture which showed no significant change when compared to the corresponding control output (Figure 3.3.c).

The output of PGF<sub>2 $\alpha$</sub>  was significantly ( $P < 0.05$ ) inhibited by W-7 (150 and 300 $\mu$ M) treatment on days 1, 2 and 3 of culture (Figure 3.3.d). Treatment with W-7 (150 and 300 $\mu$ M) significantly ( $P < 0.05$ ) increased PGE<sub>2</sub> output on all three days of culture, except for treatment with 150 $\mu$ M W-7 on day 1 of culture. The output of 6-keto-PGF<sub>1 $\alpha$</sub>  was significantly ( $P < 0.05$ ) increased by W-7 (150 and 300 $\mu$ M) on day 2 of culture and by 150 $\mu$ M W-7 on day 3 of culture (Figure 3.3.d).

Nifedipine (1 and 10 $\mu$ M) had no significant effect on the outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub> , on any day of culture, except for 1 $\mu$ M nifedipine which significantly ( $P < 0.05$ ) increased PGF<sub>2 $\alpha$</sub>  output on day 2 of culture (Figure 3.3.e). Nifedipine (100 $\mu$ M) significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output on all three days of culture, PGE<sub>2</sub> output on day 1 and day 2 of culture and 6-keto-PGF<sub>1 $\alpha$</sub>  output on day 1 of culture (Figure 3.3.e).

Verapamil (1 $\mu$ M) had no effect on the outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  on any day of culture (Figure 3.3.f). Verapamil (10 $\mu$ M and 100 $\mu$ M) significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output on day 1 of

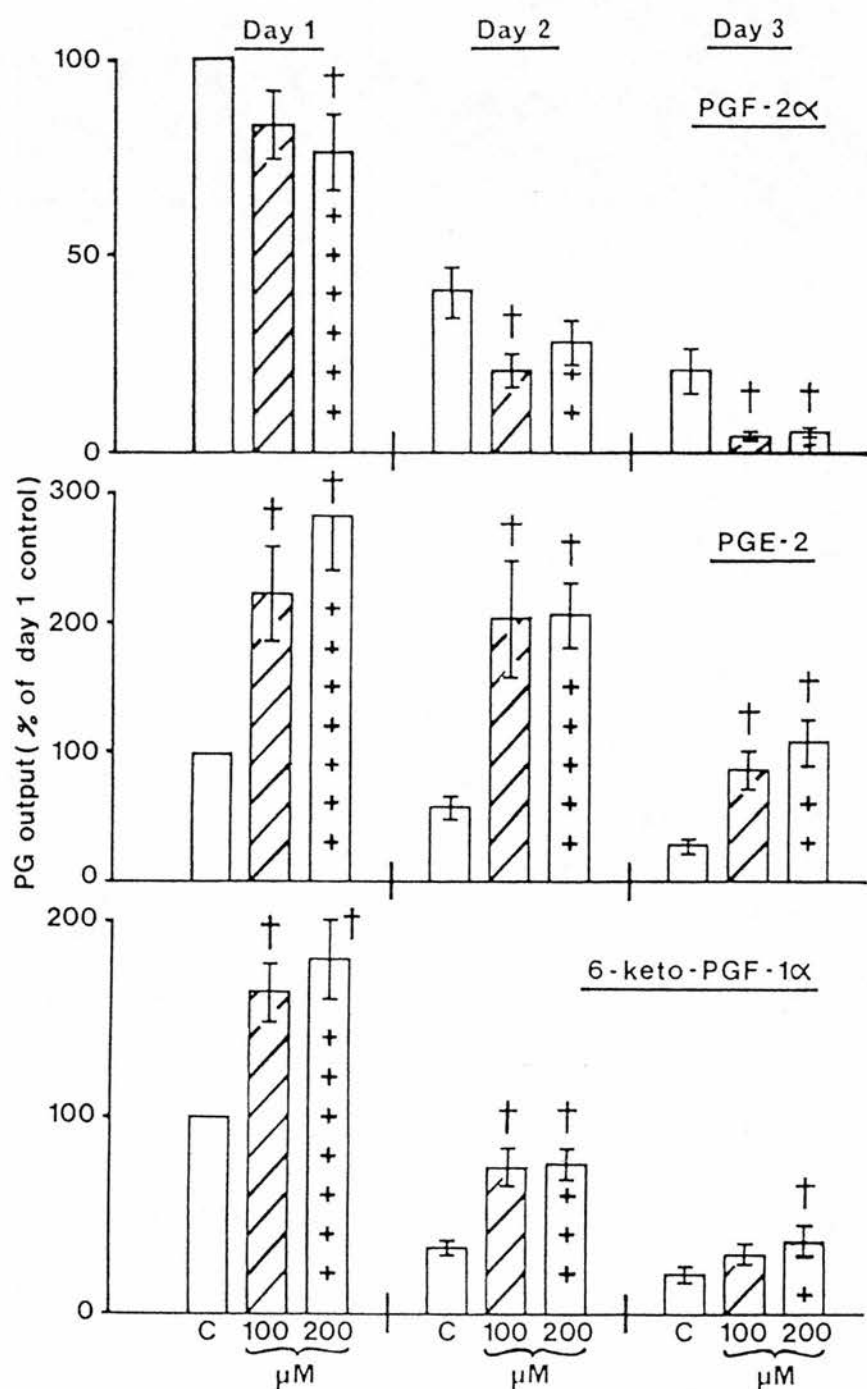


Figure 3.3.c

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF<sub>2</sub> $\alpha$ , PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca<sup>2+</sup>-containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of 100  $\mu$ M (hatched columns) and 200  $\mu$ M (crossed columns) TFP.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

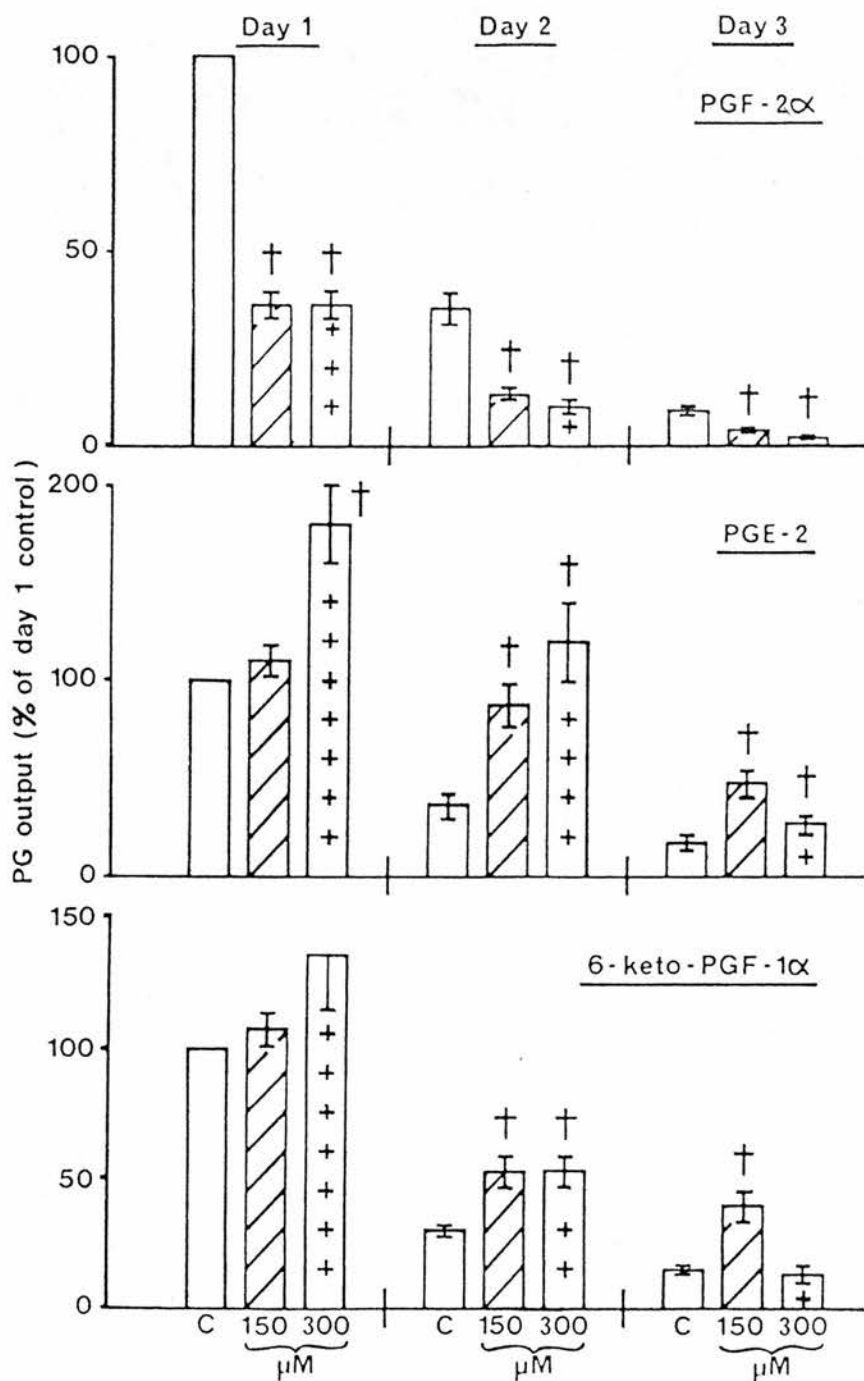


Figure 3.3.d

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of 150  $\mu$ M (hatched columns) and 300  $\mu$ M (crossed columns) W-7.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

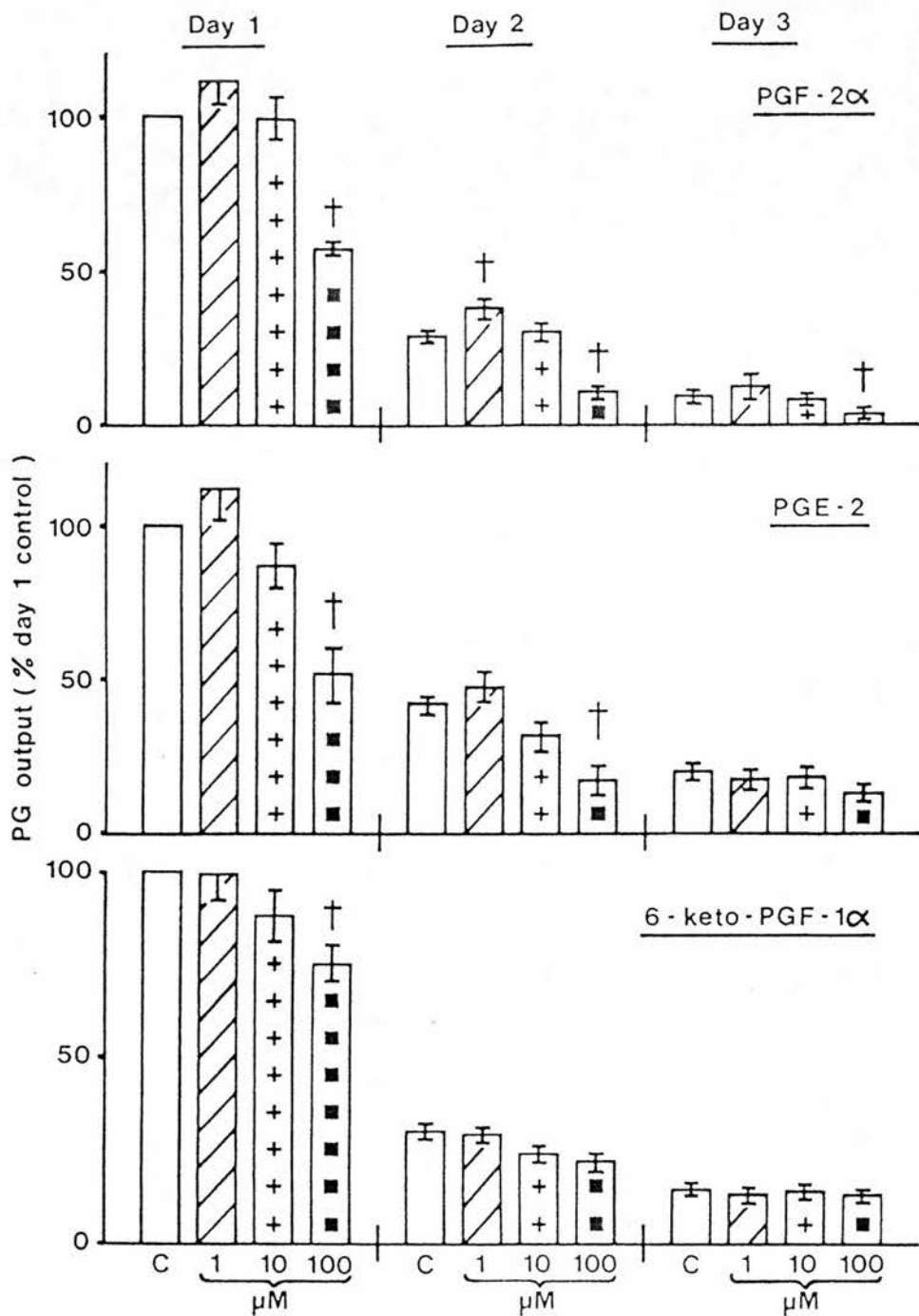


Figure 3.3.e

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF<sub>2</sub> $\alpha$ , PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca<sup>2+</sup>-containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of 1 $\mu$ M (hatched columns), 10 $\mu$ M (crossed columns) and 100 $\mu$ M (squared columns) nifedipine.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

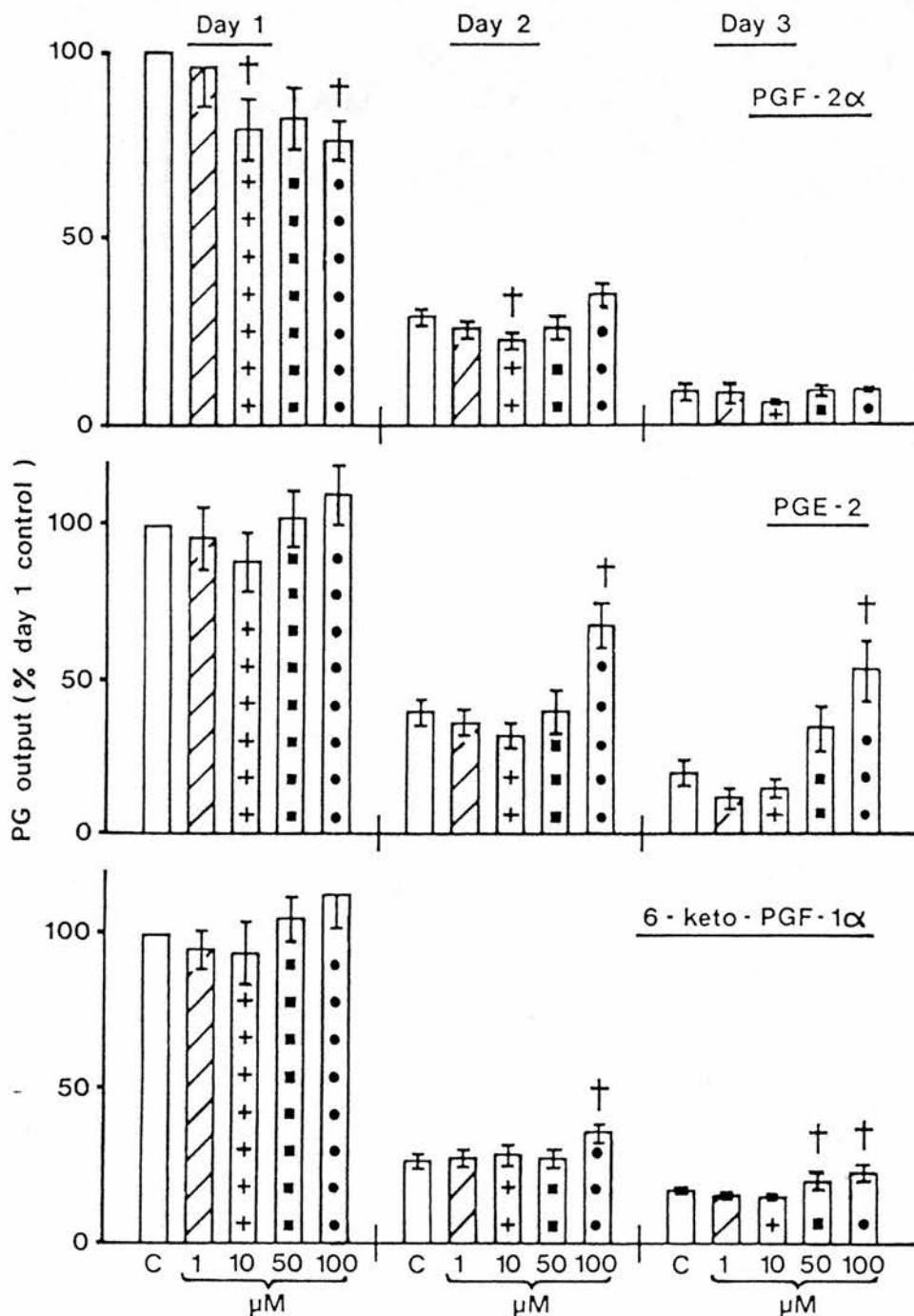


Figure 3.3.f

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -containing medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of 1 $\mu$ M (hatched columns), 10 $\mu$ M (crossed columns), 50 $\mu$ M (squared columns) and 100 $\mu$ M (circled columns) verapamil.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

culture and  $10\mu\text{M}$  verapamil significantly ( $P < 0.05$ ) reduced  $\text{PGF}_{2\alpha}$  output during day 2 of culture. The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were not significantly changed by verapamil ( $10\mu\text{M}$  and  $50\mu\text{M}$ ) on days 1, 2 and 3 of culture, except  $50\mu\text{M}$  verapamil significantly ( $P < 0.05$ ) increased 6-keto- $\text{PGF}_{1\alpha}$  output on day 3 of culture. Verapamil ( $100\mu\text{M}$ ) significantly ( $P < 0.05$ ) increased  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs on days 2 and 3 of culture (Figure 3.3.f).

TPA (1, 5 and  $25\text{ng/ml}$ ) had no significant effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  when compared to the respective control values, from either day 7 (Figure 3.3.g) or day 15 (Figure 3.3.h) cultured guinea-pig endometrium on any day of culture.

## 2. Using $\text{Ca}^{2+}$ -depleted medium

### Basal PG outputs from day 15 guinea-pig endometrium in culture

The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 guinea-pig endometrium cultured for three days in  $\text{Ca}^{2+}$ -depleted medium and in  $\text{Ca}^{2+}$ -containing medium are compared in Figure 3.3.i. The outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  on day 1 and day 2 of culture were significantly ( $P < 0.05$ ) reduced in  $\text{Ca}^{2+}$ -depleted medium. The combined outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from endometrium cultured in  $\text{Ca}^{2+}$ -depleted medium decreased by 48% and 39% on days 1 and 2, respectively. The output of 6-keto- $\text{PGF}_{1\alpha}$  was significantly ( $P < 0.05$ ) reduced by 14% on day 1 of culture in  $\text{Ca}^{2+}$ -depleted medium (Figure 3.3.i).

### Effect of various treatments on PG output

The effects of EGTA, TMB-8, W-7, nifedipine and verapamil on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 guinea-pig endometrium cultured for three days in  $\text{Ca}^{2+}$ -depleted medium are illustrated in Figures 3.3.j, 3.3.k, 3.3.l and 3.3.m, respectively.

EGTA ( $2\text{mM}$ ) significantly ( $P < 0.05$ ) reduced  $\text{PGF}_{2\alpha}$  output on days



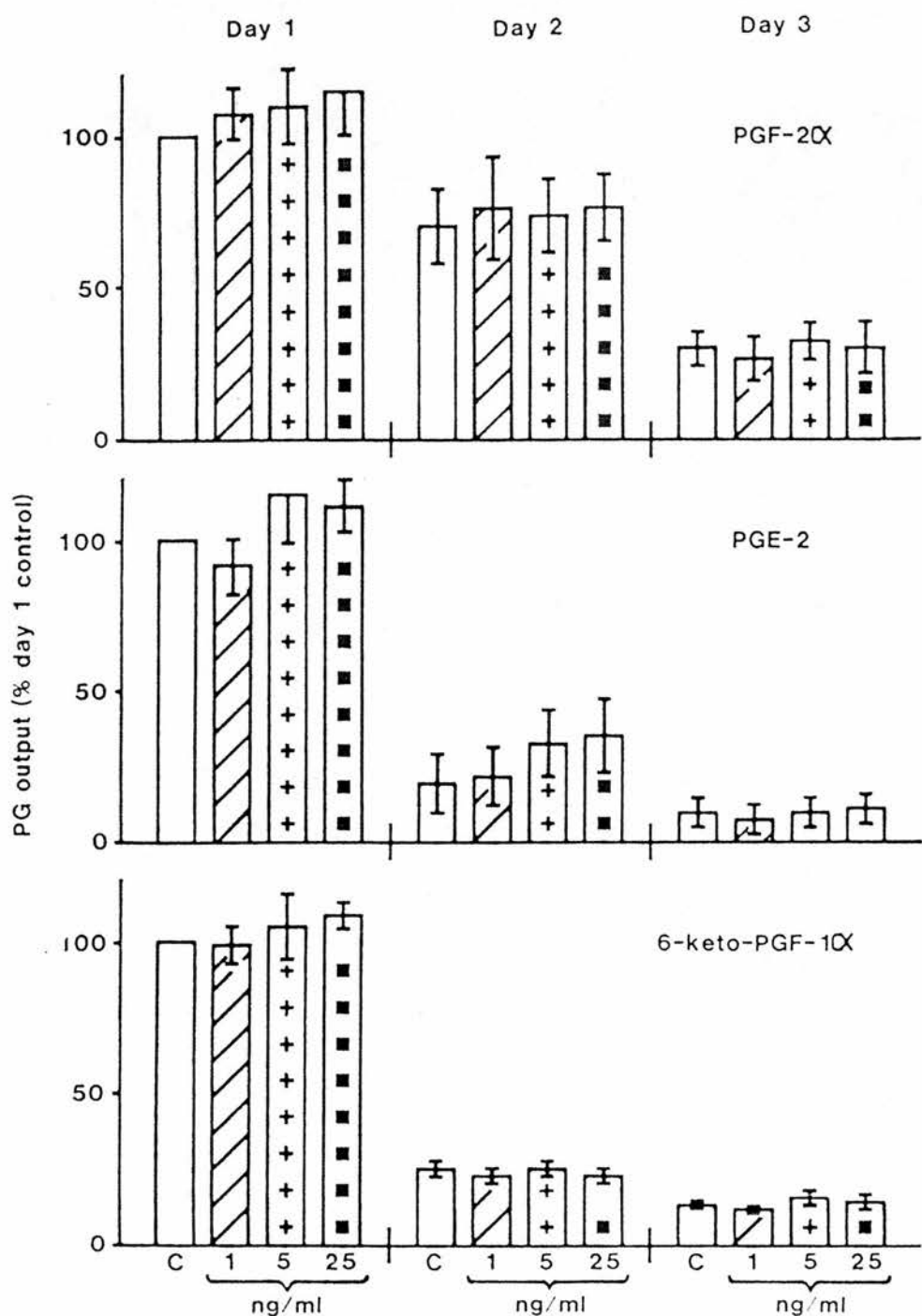


Figure 3.3.g

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium removed on day 7 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -containing medium, sampling every 24h, in the absence (controls (C); open columns) and presence of 1ng/ml (hatched columns), 5ng/ml (crossed columns) and 25ng/ml (squared columns) TPA.

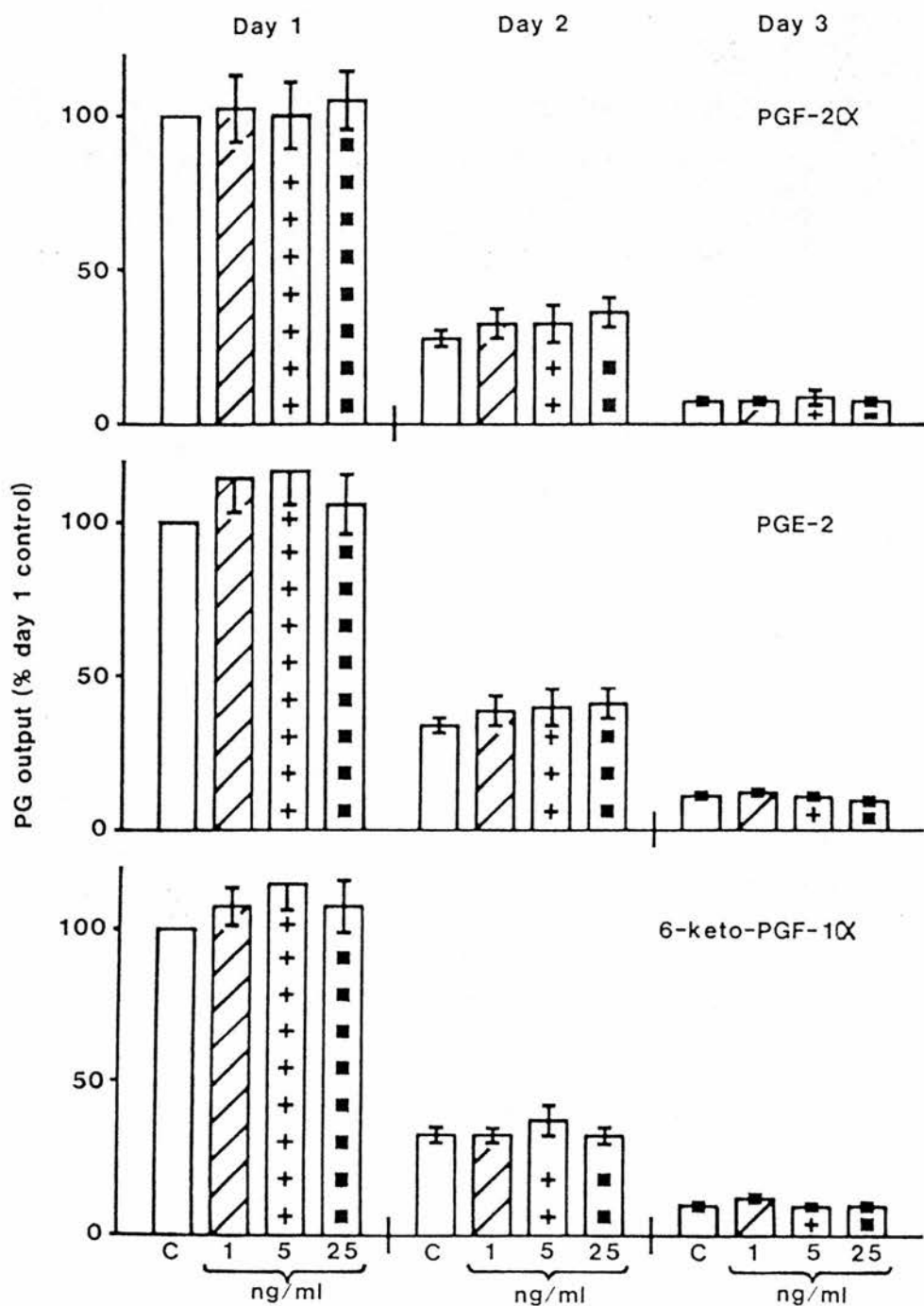


Figure 3.3.h

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -containing medium, sampling every 24h, in the absence (controls (C); open columns) and presence of 1ng/ml (hatched columns), 5ng/ml (crossed columns) and 25ng/ml (squared columns) TPA.

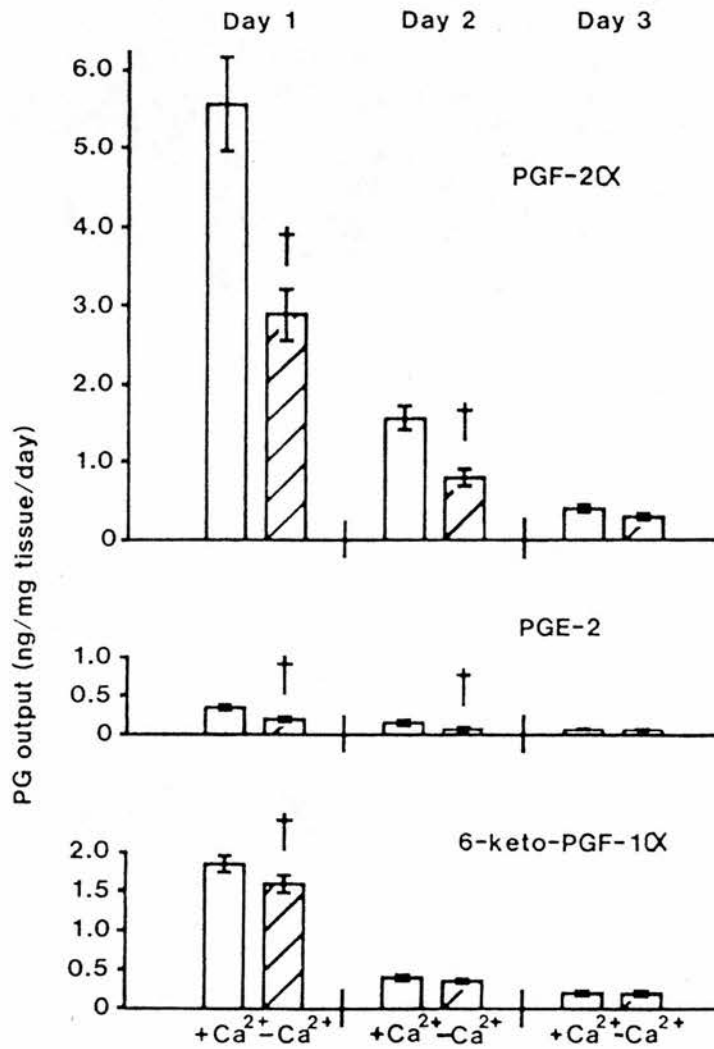


Figure 3.3.i

Mean (± s.e.m.; n = 10) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca<sup>2+</sup>-containing medium (open bars), or in Ca<sup>2+</sup>-depleted medium (hatched bars), with sampling every 24h.

† significantly (P < 0.05) lower compared to the output from endometrium cultured in Ca<sup>2+</sup>-containing medium for the same PG on the same day of culture.

1 and 2 of culture and 6-keto-PGF<sub>1α</sub> output on day 1 of culture (Figure 3.3.j). EGTA (2mM) significantly ( $P < 0.05$ ) increased 6-keto-PGF<sub>1α</sub> output on days 2 and 3 of culture (Figure 3.3.j).

The output of PGF<sub>2α</sub> was significantly ( $P < 0.05$ ) reduced on days 1 and 2, and the output of PGE<sub>2</sub> was significantly ( $P < 0.05$ ) increased on day 1 and day 2 of culture by 100μM TMB-8 treatment (Figure 3.3.k). The output of 6-keto-PGF<sub>1α</sub> was significantly ( $P < 0.05$ ) increased by 100μM TMB-8 on day 2 and day 3 of culture (Figure 3.3.k).

The output of PGF<sub>2α</sub> was significantly ( $P < 0.05$ ) inhibited on day 1 of culture by 150μM W-7 (Figure 3.3.l). W-7 significantly ( $P < 0.05$ ) increased the output of PGE<sub>2</sub> on all three days of culture, and significantly ( $P < 0.05$ ) increased the output of 6-keto-PGF<sub>1α</sub> on days 2 and 3 of culture (Figure 3.3.l).

Nifedipine (100μM) significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs from endometrium cultured in Ca<sup>2+</sup>-depleted medium during day 1 of culture, significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub> and PGE<sub>2</sub> outputs during day 2 of culture, and significantly ( $P < 0.05$ ) inhibited PGE<sub>2</sub> output during day 3 of culture (Figure 3.3.m). Verapamil (100μM) significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub> output during day 1 of culture and significantly ( $P < 0.05$ ) increased PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs on days 2 and 3 of culture (Figure 3.3.m).

## CONCLUSIONS

The output of PGF<sub>2α</sub>, which is high from guinea-pig endometrium on day 15 of the oestrous cycle and cultured in Ca<sup>2+</sup>-containing medium, was reduced when cultured in Ca<sup>2+</sup>-depleted medium. Endometrial PGF<sub>2α</sub> output from day 15 cultured endometrium was also reduced when cultured in Ca<sup>2+</sup>-containing medium and treated with EGTA

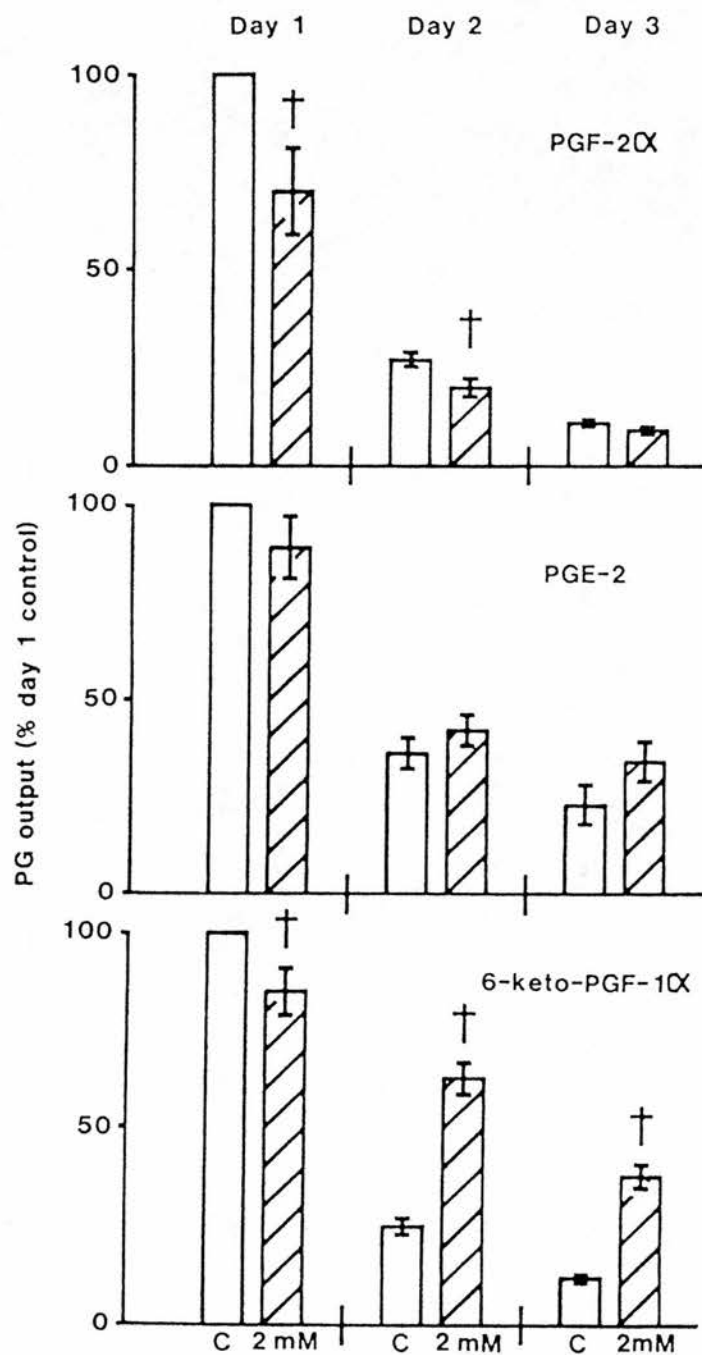


Figure 3.3.i

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_1\alpha$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -depleted medium, with sampling every 24h, in the absence (controls (C); open columns) and presence (hatched columns) of 2mM EGTA.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

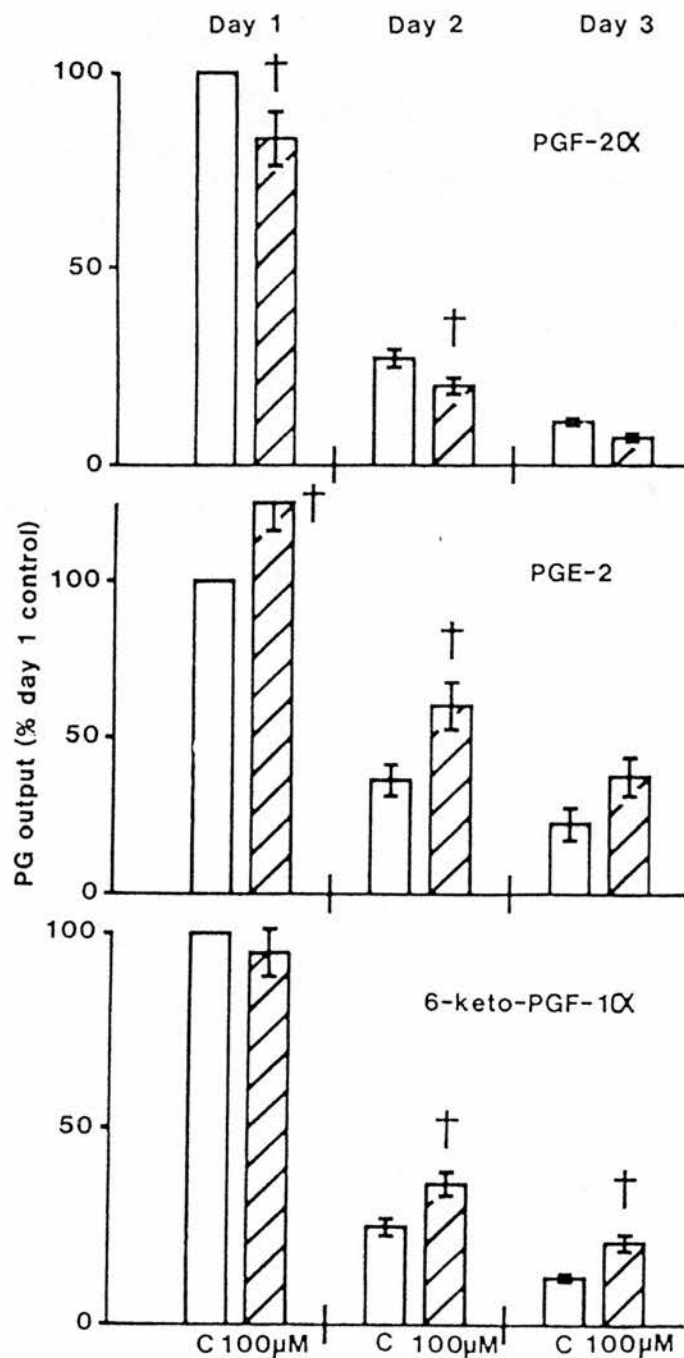


Figure 3.3.k

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in  $\text{Ca}^{2+}$ -depleted medium, with sampling every 24h, in the absence (controls (C); open columns) and presence (hatched columns) of  $100\mu\text{M}$  TMB-8.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

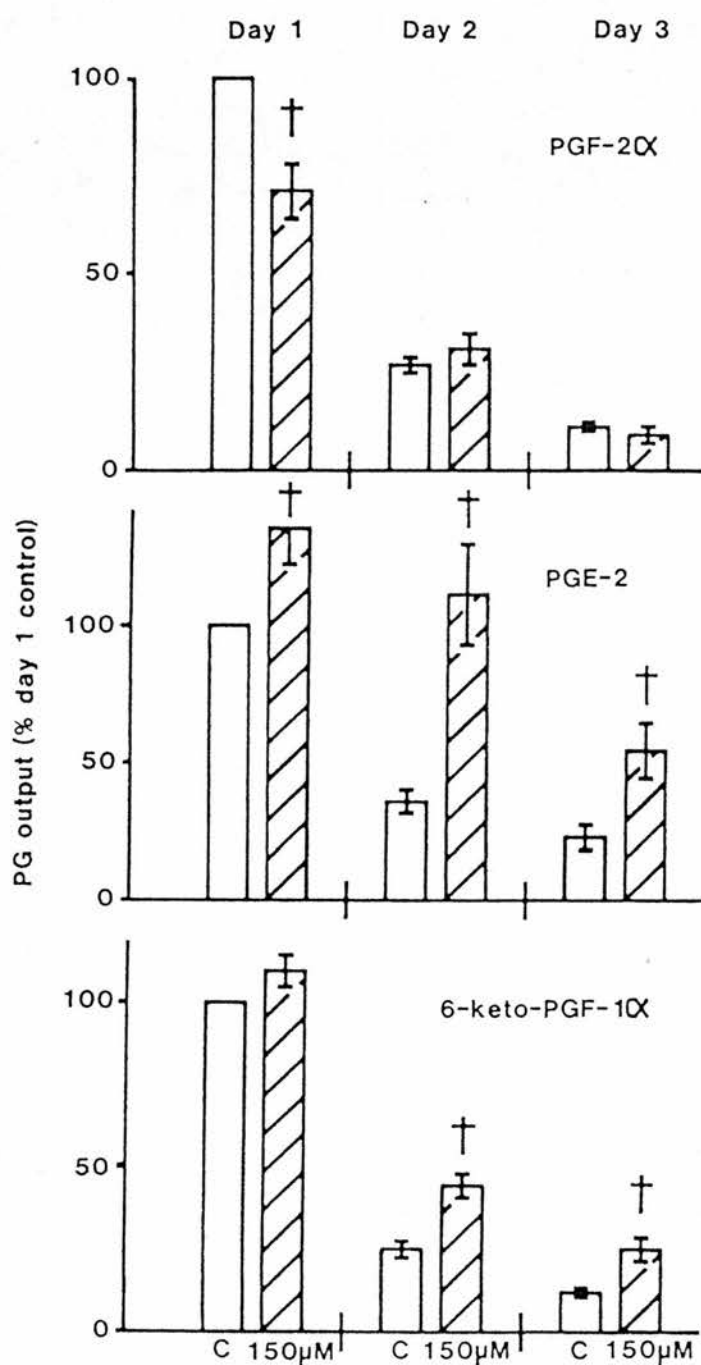


Figure 3.3.1

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in  $\text{Ca}^{2+}$ -depleted medium, with sampling every 24h, in the absence (controls (C); open columns) and presence (hatched columns) of  $150\mu\text{M}$  W-7.

† significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.

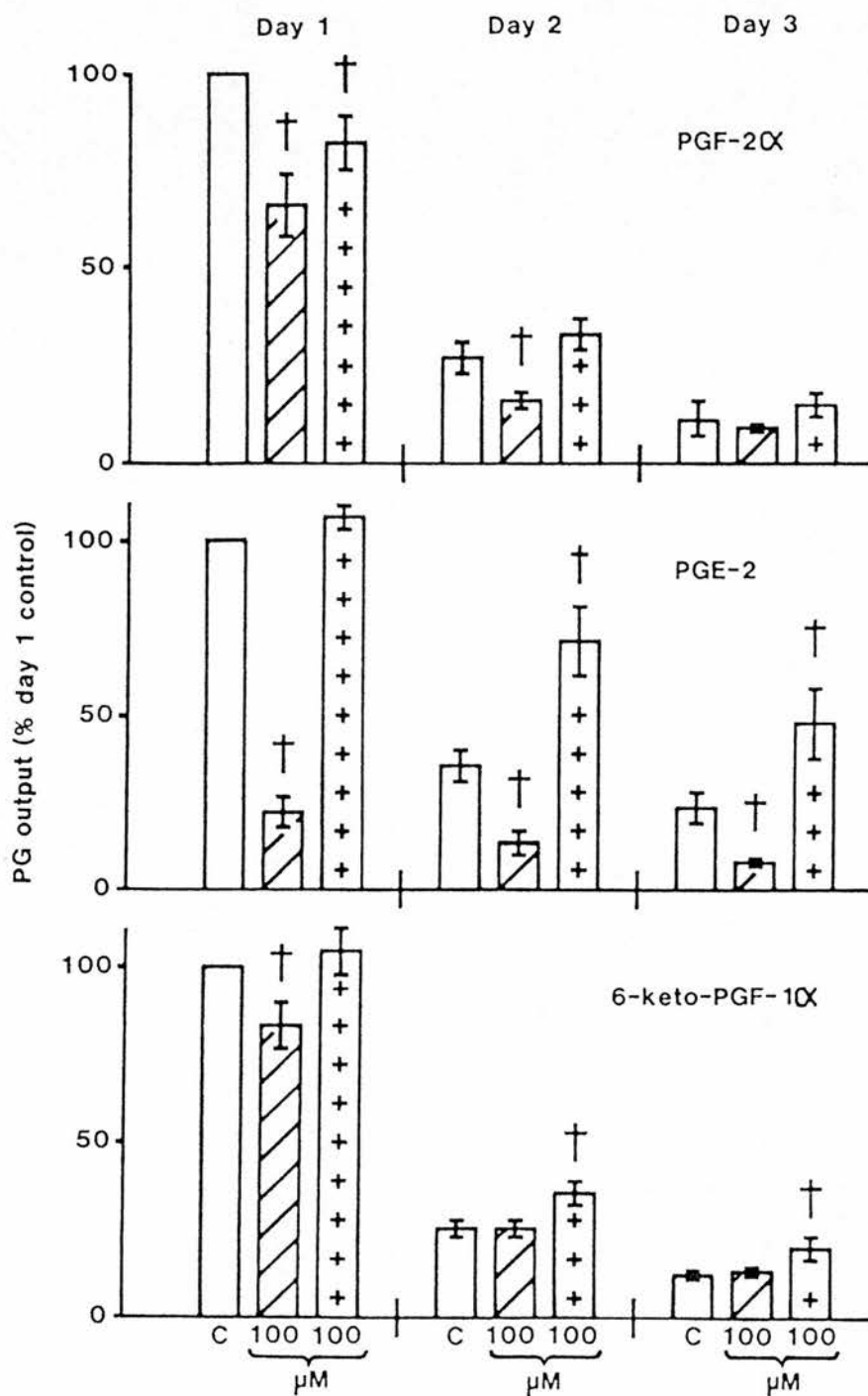


Figure 3.3.m

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs (expressed as % of day 1 control (C) output for the same PG) of PGF $_{2\alpha}$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for three days in Ca $^{2+}$ -depleted medium, with sampling every 24h, in the absence (controls (C); open columns) and presence of 100  $\mu$ M nifedipine (hatched columns) and 100  $\mu$ M verapamil (crossed columns).  
 † significantly ( $P < 0.05$ ) different compared to the control value for the same PG on the same day of culture.



(which chelates  $\text{Ca}^{2+}$ ), and with the intracellular  $\text{Ca}^{2+}$  antagonist, TMB-8. Treatment with EGTA and TMB-8 also caused a further small reduction in  $\text{PGF}_{2\alpha}$  output from endometrium cultured in  $\text{Ca}^{2+}$ -depleted medium. These findings show that the high output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium in culture is dependent on the presence of  $\text{Ca}^{2+}$ , and that the  $\text{Ca}^{2+}$  required is primarily extracellular in source.

The high output of  $\text{PGF}_{2\alpha}$  from day 15 cultured endometrium was reduced by treatment with the calmodulin inhibitors W-7 and, to a lesser degree, TFP when cultured in  $\text{Ca}^{2+}$ -containing medium, and also by W-7 treatment in  $\text{Ca}^{2+}$ -depleted medium. This suggests that calmodulin is necessary for maintenance of the high output of  $\text{PGF}_{2\alpha}$  from day 15 guinea-pig endometrium in culture.

At low concentrations of nifedipine (1 and  $10\mu\text{M}$ ) and of verapamil ( $1\mu\text{M}$ ) (concentrations which normally block voltage-dependent calcium channels), there was no change in  $\text{PGF}_{2\alpha}$  output from cultured day 15 endometrium. However, at higher concentrations of nifedipine ( $100\mu\text{M}$ ) and verapamil (10 and  $100\mu\text{M}$ ) there was some inhibitory effect on  $\text{PGF}_{2\alpha}$  output. This inhibitory action on  $\text{PGF}_{2\alpha}$  output was also present in  $\text{Ca}^{2+}$ -depleted medium, suggesting that the effect was due to an intracellular action of the compounds and was not due to the blocking of voltage-dependent calcium channels.

The removal of extracellular  $\text{Ca}^{2+}$  inhibited the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , but these outputs were inhibited to a lesser degree than the inhibition of  $\text{PGF}_{2\alpha}$  output. However, TMB-8, TFP, W-7, verapamil and, to a lesser extent, EGTA all stimulated  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs, and therefore have apparently opposing actions on the production of different PGs. Nifedipine at the highest concentration used ( $100\mu\text{M}$ ) did not exhibit this opposing action on the production of different PGs, but exhibited an inhibitory effect on all

PGs.

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were not changed by treatment with TPA; these findings indicate that protein kinase C is not involved in the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis.

## DISCUSSION

Endometrial  $\text{PGF}_{2\alpha}$  output was inhibited by culturing in  $\text{Ca}^{2+}$ -depleted medium, and also in  $\text{Ca}^{2+}$ -containing medium with the addition of EGTA. This suggests that extracellular calcium is necessary for the stimulation of  $\text{PGF}_{2\alpha}$  output. Treatment with TMB-8, an intracellular calcium antagonist, also exhibited an inhibitory action of  $\text{PGF}_{2\alpha}$  output. This action of TMB-8 may either be due to an inhibitory effect on extracellular calcium that enters the cell, by making it unavailable for its  $\text{PGF}_{2\alpha}$  stimulating activity, or an effect on intracellular calcium already present in the cell. As TMB-8 caused a further slight inhibition of  $\text{PGF}_{2\alpha}$  output in  $\text{Ca}^{2+}$ -depleted medium, TMB-8 may be acting by a combination of both mechanisms. Scharff and Foder (1984) reported that the effects of TMB-8 are not totally specific on intracellular calcium stores. At high concentrations, TMB-8 decreased calmodulin sensitivity of red blood cell  $\text{Ca}^{2+}$ -ATPase, a model which is devoid of intracellular  $\text{Ca}^{2+}$  stores, and calmodulin has also been implicated in the control of  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus. Also TMB-8 altered phosphatidylinositol labelling, an effect usually related directly to effects on the plasma membrane (Uchida, Filburn and Sacktor, 1984), suggesting TMB-8 may inhibit membrane bound  $\text{PLA}_2$  directly. Donowitz, Cusolito and Sharp (1986) showed a maximal effect of TMB-8 ( $50\mu\text{M}$ ) in increasing active  $\text{Na}^+$  and  $\text{Cl}^-$  absorption by rabbit ileum. They reported that the effects of TMB-8 at this order of concentration

appeared to be consistent with an action to trap calcium within intracellular stores, and TMB-8 did not appear to change  $\text{Ca}^{2+}$  entry. The concentrations of TMB-8 used are similar to those examined in this thesis, and it appears that other effects of TMB-8 on  $\text{PGF}_{2\alpha}$  output (e.g. action via calmodulin and via direct membrane effect) may not be significant.

EGTA treatment also caused a further slight inhibition of  $\text{PGF}_{2\alpha}$  output from endometrium cultured in  $\text{Ca}^{2+}$ -depleted medium. Therefore, EGTA may possess both an intracellular and extracellular effect, or its action may be due to a chelation of any residual calcium in the extracellular medium, and any calcium that is released from the tissue during culture. However, structural changes occur in HeLa and human fibroblast cells, which manifest themselves as an increase in the number of microvilli and the appearance of surface blebs when treated with EDTA (Moskalewski and Thyberg, 1981). EDTA is a close chemical analogue of EGTA so a contributing factor in the inhibitory effect of EGTA in both  $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -depleted medium may be due to non-specific structural changes in the endometrial cells.

The high basal output of  $\text{PGF}_{2\alpha}$  from day 15 guinea-pig endometrium was inhibited by the calmodulin inhibitors W-7 and, to a lesser extent, TFP, suggesting that calmodulin is involved in the stimulation of  $\text{PLA}_2$  activity and hence PG synthesis. However, whether the interaction of calmodulin with  $\text{PLA}_2$  is necessary to stimulate  $\text{PLA}_2$  activity is controversial. Withnall and Brown (1982) reported that several drugs that act as calmodulin antagonists, including TFP, inhibit pancreatic  $\text{PLA}_2$  activity directly. They proposed that calmodulin was not involved in the stimulation of pancreatic  $\text{PLA}_2$ . However, a study by Gerritsen, Nganele and Rodrigues (1987) reported that TFP inhibited the activity of porcine

pancreatic PLA<sub>2</sub>. Ballou and Cheung (1983) also reported that calmodulin demonstrated no stimulatory activity on purified platelet PLA<sub>2</sub>. Also, calmodulin exhibited no stimulatory effect on membrane-bound or purified PLA<sub>2</sub> from mitochondria when an endogenous substrate was used ([<sup>14</sup>C]-ethanolamine-labelled mitochondria); Calmodulin exhibited only a small (20%) stimulatory action on PLA<sub>2</sub> using an endogenous substrate (deWinter, Korpancova and Van den Bosche, 1984). Watanabe, Hashimoto, Teramoto, Kuma, Naito and Oka (1986) showed that platelet PLA<sub>2</sub> was stimulated by acidic phospholipids (such as phosphatidic acid and phosphatidylserine) but not by calmodulin, in a calcium-dependent manner. The calmodulin inhibitors TFP and W-7 inhibited this stimulation of PLA<sub>2</sub> by an inhibition of calcium binding to the acidic phospholipids.

The affinity <sup>to calmodulin</sup> of phenothiazines, of which TFP is a derivative, is related to their hydrophobicity, and they possess greater affinity in the presence of Ca<sup>2+</sup>, an effect which is probably due to modified exposure of hydrophobic areas in the Ca<sup>2+</sup>-bound conformation of calmodulin. A calcium-induced conformational change of calmodulin has been detected by <sup>1</sup>H-NMR spectroscopy (Ikura, Hiraoki, Hikichi, Mikuni, Yazawa and Yagi, 1983). The interaction of TFP with calmodulin was investigated by Dalgarno, Klevit, Levine, Scott, Williams, Gergely, Grabarek, Leavis, Grand and Drabikowski (1984), and it was determined that the TFP-induced inhibition of the biological activity of calmodulin is apparently due to conformational restrictions caused by the binding of TFP. They proposed that TFP restricts the conformations of calmodulin in which (a) Ca<sup>2+</sup> is released and (b) calmodulin interacts with its partner protein/enzyme. Sutoo, Akiyama, Fujii and Matsushita (1986) demonstrated that the conformational changes of calmodulin produced by W-7, when calmodulin

was fully bound with  $\text{Ca}^{2+}$ , were similar to TFP, but they suggested that W-7 may also possess a further binding site. From this evidence, both TFP and W-7 bind to calmodulin and inhibit its biological effect. Phospholipase  $\text{A}_2$  enzymes have been demonstrated to be regulated by several mechanisms, and not every  $\text{PLA}_2$  has been demonstrated to require calmodulin. Extracellular pancreatic  $\text{PLA}_2$  has a much higher enzymic activity than cellular  $\text{PLA}_2$ , and also platelet  $\text{PLA}_2$  may be controlled by other mechanisms.

Nifedipine and verapamil at concentrations that normally block voltage-dependent calcium channels (Godfraind, Miller and Wibo, 1986) had no effect on basal  $\text{PGF}_{2\alpha}$  output from cultured day 15 endometrium. However, nifedipine ( $100\mu\text{M}$ ) had a marked inhibitory effect and verapamil ( $10\text{-}100\mu\text{M}$ ) also exhibited an inhibitory action on basal  $\text{PGF}_{2\alpha}$  output, but this effect was smaller and was only significant sometimes. This inhibitory effect of verapamil and nifedipine on  $\text{PGF}_{2\alpha}$  output was also present in  $\text{Ca}^{2+}$ -depleted medium, indicating that it is not due to a voltage-dependent calcium channel blocking action and that extracellular calcium is not involved. In a NMR study examining interactions with calmodulin, the verapamil derivative, D600, bound to and inhibited the action of calmodulin (Andersson, Drakenberg, Thulin and Forsen, 1983). Nifedipine ( $10\text{-}440\mu\text{M}$ ) also partially inhibited calmodulin activated cAMP phosphodiesterase activity (Minocherhomjee and Roufogalis, 1984). Calmodulin activates calmodulin-deficient phosphodiesterase and Schlondorff and Satriano (1985) demonstrated that this activation was inhibited by the verapamil derivative, D600 ( $100\mu\text{M}$ ). This inhibitory effect was overcome by increasing the calmodulin concentration. Nifedipine ( $15\text{-}30\mu\text{M}$ ) also inhibited phosphodiesterase activity but this inhibitory effect was not overcome by adding calmodulin. This suggests that

verapamil, but not nifedipine acts via the inhibition of calmodulin. This may be further and indirect evidence that implicates calmodulin as a mediator of endometrial  $\text{Ca}^{2+}$ -stimulated  $\text{PGF}_{2\alpha}$  synthesis. Furthermore, Chang, Blazek and Carlson (1987) proposed that nifedipine may inhibit  $\text{PLA}_2$  activity directly, when using semi-purified preparations of  $\text{PLA}_2$  obtained from platelets and mouse peritoneal macrophages, an action that is independent of its channel blocking activity. However, they appeared not to have considered the report of Hope, Welton and Swislocki (1986) that  $\text{PLA}_2$  preparations may be contaminated with calmodulin. Therefore, this may not be a direct inhibitory effect of nifedipine on  $\text{PLA}_2$  but rather via an action on calmodulin. Godfraind, Miller and Wibo (1986) concluded that dihydropyridine derivatives (e.g. nifedipine) have a very low affinity for calmodulin and only at high concentrations, as used in this present study was there any interaction with calmodulin. Erdreich and Rahamimoff (1987) proposed that verapamil at high concentrations (up to  $200\mu\text{M}$ ) binds non-specifically to phospholipid layers and this effect may cause intracellular effects on membrane-bound proteins. Therefore, verapamil (at the highest concentration used in this study) may inhibit  $\text{PLA}_2$ , a membrane bound protein, by a direct effect via the membrane.

Paradoxically, TMB-8, TFP, W-7, verapamil, and also but to a lesser extent, EGTA stimulated the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 cultured endometrium. In previous studies (Poyser, 1985a,b), TMB-8, TFP and W-7 caused a small stimulation of the basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the guinea-pig uterus superfused in vitro. TMB-8, TFP and W-7 appear to possess opposing actions on PG synthesis and release, although the inhibitory action on  $\text{PGF}_{2\alpha}$  is predominant in the tissue culture studies. Verapamil and

EGTA also exhibit similar opposing stimulatory and inhibitory effects on PG output, whereas nifedipine was the only compound investigated in this study which exhibited solely an inhibitory action on the synthesis of all PGs. Nifedipine may therefore possess a direct inhibitory effect on PLA<sub>2</sub> (Chang, Blazek and Carlson, 1987). A similar stimulatory effect on the synthesis of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> by calcium channel blockers (including verapamil) has also been reported in cultured rat cardiac myocytes (Escoubet, Griffaton, Samuel and Lechat, 1986). This effect was suggested to be independent of calcium channel blocking activity. Interestingly, TFP exhibited a greater stimulatory action on PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs than the other compounds investigated, which may explain its lesser inhibitory action on PGF<sub>2α</sub> output from cultured day 15 guinea-pig endometrium. Indeed, TFP has been demonstrated to stimulate arachidonic acid release from phosphatidylcholine and phosphatidylethanolamine in guinea-pig macrophages, and this is presumably available for PG synthesis (Takenawa, Homma and Nagai, 1982).

Lipocortin inhibits PLA<sub>2</sub> activity (Blackwell and Flower, 1983), and a lipocortin-like protein in platelets is phosphorylated by protein kinase C, which causes inactivation of its PLA<sub>2</sub> inhibitory action (Touqui, Rothhut, Shaw, Fradin, Vargaftig and Russo-Marie, 1986). However PG outputs were unaltered by TPA treatment of day 7 and 15 guinea-pig endometrium in culture, so PLA<sub>2</sub> activity in this tissue is not normally controlled by protein kinase C-inactivated, lipocortin-like protein. Kojima, Kojima and Rasmussen (1985) investigated the effect of TMB-8 on isolated adrenal glomerulosa cells and they showed that TMB-8 inhibited the activity of protein kinase C directly. However, in this study TPA has no effect on PG production by the guinea-pig endometrium in culture and so this secondary effect



of TMB-8 on protein kinase C is not significant.

Overall, the results in this section have shown that  $\text{Ca}^{2+}$  is required for the high output of  $\text{PGF}_{2\alpha}$  from cultured, day 15 guinea-pig endometrium, and also, but to a lesser extent, for the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ . The primary source of this  $\text{Ca}^{2+}$  is extracellular in origin but the  $\text{Ca}^{2+}$  does not enter the cells by voltage-dependent  $\text{Ca}^{2+}$  channels. Calmodulin has also been implicated in the control of  $\text{PGF}_{2\alpha}$  output, but the control of  $\text{PLA}_2$  by a lipocortin-like protein appears not to be involved.



### 3.4 SYNTHESIS OF CELLULAR AND SECRETED PROTEINS BY GUINEA-PIG

#### ENDOMETRIUM IN CULTURE

##### INTRODUCTION

Oestradiol acting on a progesterone-primed uterus appears to be the stimulus for the increase in PGF<sub>2α</sub> output towards the end of the oestrous cycle in the guinea-pig (Blatchley and Poyser, 1974; Poyser, 1983b). These ovarian steroids have well-documented stimulatory effects upon tissue metabolism, differentiation and growth in the female reproductive tract. The primary pathway by which oestradiol and progesterone exert their effects is by stimulating the production of specific RNAs that carry the information required for the synthesis of new proteins which produce the physiological changes (Bremner and West, 1975). Both oestradiol and progesterone have been shown to stimulate protein synthesis in endometrium, in culture, from the cow (Bartol, Roberts, Bazer and Thatcher, 1985), human (Strinden and Shapiro, 1983), rabbit (Rajkumar, Bigsby, Lieberman and Gershenson, 1983), rat (Komm, Keeping, Sabogal and Lyttle, 1985), and sheep (Miller, Murphy and Stone, 1975), and also in the foetal guinea-pig uterus (Sumida and Pasqualini, 1981). Poyser (1979) has shown that the intra-uterine injection of actinomycin D, an inhibitor of DNA-dependent RNA synthesis inhibits PGF<sub>2α</sub> production by homogenates of the guinea-pig uterus. It was proposed that changes in protein synthesis (controlled by ovarian steroids) could control PG production by the guinea-pig uterus.

This study has investigated the incorporation of [<sup>3</sup>H]-leucine into cellular and secreted proteins synthesised by the guinea-pig endometrium removed on day 7 and day 15 of the oestrous cycle and cultured for up to 24h. The aims of the experiment are:-

(a) to examine whether de novo protein synthesis occurs in

endometrium in culture.

(b) if protein synthesis does occur, to investigate the rates of synthesis of cellular and secreted proteins by day 7 and day 15 cultured endometrium, and whether these rates change over 24h.

(c) if protein synthesis does occur, to determine a suitable period of culture during which the effects of protein synthesis inhibitors and steroids on protein synthesis can be investigated (see sections 3.5 and 3.6, respectively).

## METHODS

Five day 7 and five day 15 guinea-pigs were killed and their uteri removed in preparation for tissue culture (section 2.2.1). For each uterus, endometrium was separated from the myometrium and six Petri dishes were prepared for tissue culture, each dish containing between 30 and 60mg endometrium (section 2.2.5). Each dish also contained 10 $\mu$ Ci [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml) according to section 2.4 (i.e. the method for the measurement of protein synthesis). These dishes were placed in a modified Kilner jar which was gassed and incubated according to section 2.2.5. Immediately after the jar was placed in the incubator, 30-60mg of the remaining endometrium was placed in another Petri dish (which also contained 10 $\mu$ Ci [ $^3$ H]-leucine) according to the tissue culture method above. The endometrium in the dish was washed with the [ $^3$ H]-leucine-containing medium using a Pasteur pipette and was incubated for 2 min at room temperature. The endometrium was removed and weighed accurately. Then the procedure for measuring [ $^3$ H]-leucine incorporation into cellular and secreted proteins was followed for this tissue and the medium in the dish (section 2.4). This endometrium and culture medium measured the non-specific binding of [ $^3$ H]-leucine that was retained in the multiple

stage process to measure [ $^3\text{H}$ ]-leucine incorporation into cellular and secreted proteins, respectively.

After 1h, 2h, 4h, 8h, 12h and 24h, one dish was removed from the Kilner jar at each time, and the remaining dishes were replaced in the jar which was then regassed and incubated as specified above (except at 24h). The amounts of [ $^3\text{H}$ ]-leucine incorporated into endometrial cellular and secreted proteins was measured as described in section 2.4 from each of the dishes as they were removed.

#### Analysis of results

All results were expressed as cpm/mg tissue/ $10^6$  standard counts. For each set of dishes, the respective non-specific binding counts (measured after 2 min) for cellular and secreted proteins were subtracted from their corresponding incorporated counts measured between 1h and 24h. The line of best fit for [ $^3\text{H}$ ]-leucine incorporation into total secreted and cellular proteins on day 7 and day 15 was calculated by linear regression analysis ( $r > 0.98$  in all cases). Student's 't' test was used to compare the amounts of [ $^3\text{H}$ ]-leucine incorporated into cellular and secreted proteins by cultured endometrium removed on day 7 and day 15 of the oestrous cycle after each period in culture.

#### RESULTS

The incorporation of [ $^3\text{H}$ ]-leucine into newly synthesised cellular and secreted proteins by guinea-pig endometrium removed on day 7 and day 15 of the oestrous cycle and cultured for up to 24h is shown in Figure 3.4.a. The incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted proteins during the 24h culture period was linear by both day 7 and day 15 endometrium. There were no significant differences in the amounts of [ $^3\text{H}$ ]-leucine incorporated

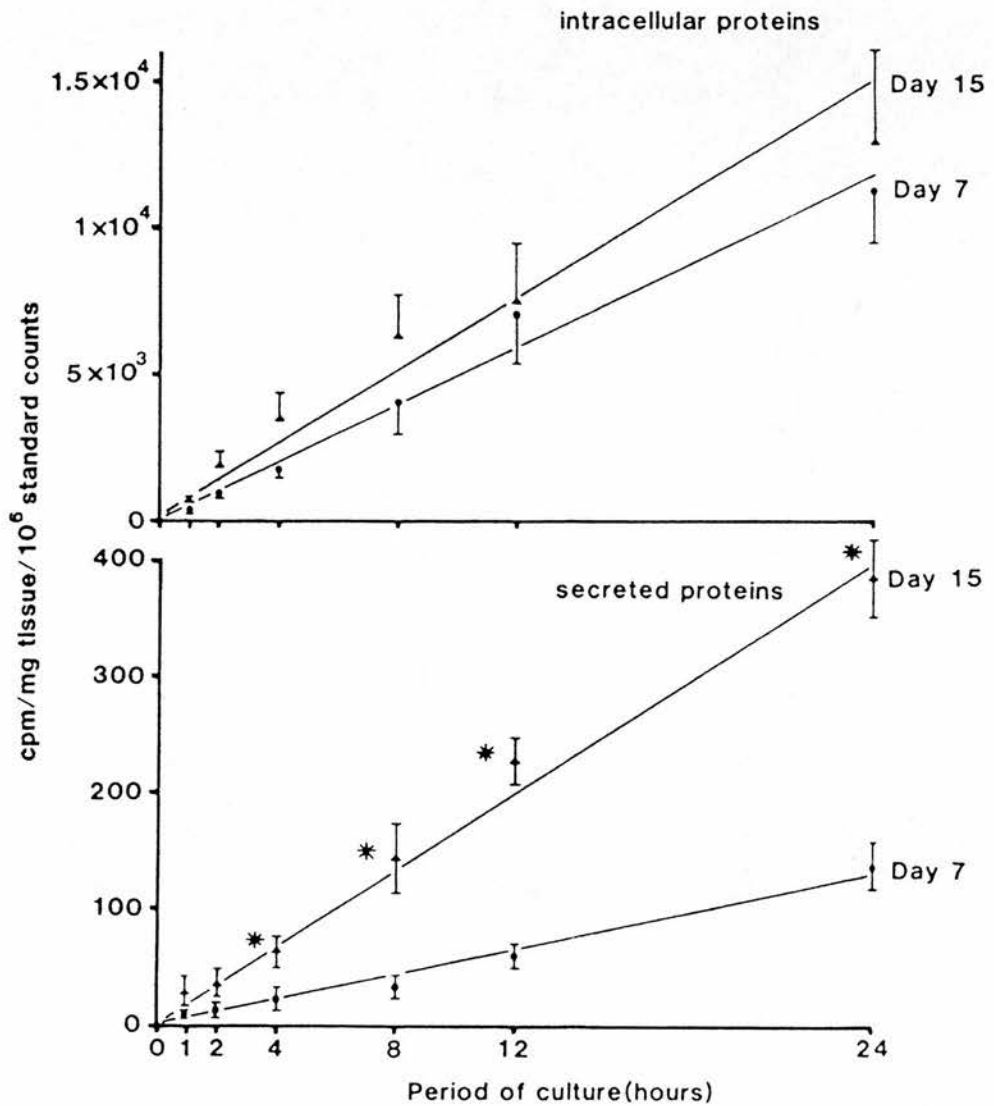


Figure 3.4.a

Time course of [<sup>3</sup>H]-leucine incorporation into cellular and secreted proteins by day 7 and day 15 guinea-pig endometrium in culture for up to 24h (mean  $\pm$  s.e.m.; n = 5).

\* significantly (P < 0.05) higher than [<sup>3</sup>H]-leucine incorporation into proteins secreted by day 7 endometrium after the same period in culture.

into cellular proteins by day 7 and day 15 endometrium at any time during the culture period. However, after 4h and for the remainder of the 24h culture period, significantly ( $P < 0.05$ ) more [ $^3\text{H}$ ]-leucine was incorporated into secreted proteins by day 15 endometrium than by day 7 endometrium.

## CONCLUSIONS

Radiolabelled leucine was incorporated into cellular and secreted proteins by the guinea-pig endometrium in culture, which showed that de novo protein synthesis occurred in this preparation. Fresh protein synthesis of both total cellular and secreted proteins occurred at a constant rate during the 24h period in culture, which is shown by the linear plots of [ $^3\text{H}$ ]-leucine incorporation. There was no difference in the rates of synthesis of cellular proteins between day 7 and day 15 endometrium during 24h in culture. However, there was a significantly ( $P < 0.05$ ) higher rate of synthesis of secreted proteins by the day 15 endometrium. After 24h of culture, the amount of secreted protein synthesised by day 15 endometrium was 2.8-fold greater than by day 7 endometrium. From these data, the optimum period for culturing endometrium and treating with various factors to investigate changes in the rate of protein synthesis is 24h. The rate of [ $^3\text{H}$ ]-leucine incorporation into both cellular and secreted proteins by day 7 and day 15 endometrium is constant during 24h in culture (demonstrated by linear plot), and by using longer periods of culture, it is easier to determine changes in the rates of protein synthesis.

## DISCUSSION

This study has shown that fresh protein is synthesised by the

guinea-pig endometrium in culture, and that the rates of synthesis of cellular and secreted protein are linear during the 24h culture period. This is in agreement with Strinden and Shapiro (1983) who showed a linear incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted proteins synthesised by the human proliferative endometrium in organ culture for at least 10h and then up to 20h. Bartol, Roberts, Bazer and Thatcher (1985) have shown that the bovine endometrium in culture can be maintained successfully for periods of at least 24h for recovery of proteins synthesised and released into the medium. This is in agreement with the findings presented here, where the rates of protein synthesis were constant for 24h. Bartol, Roberts, Bazer and Thatcher (1985) also suggested that endometrial tissues in this short term explant culture reflect the same metabolic state of the tissues *which* existed in vivo prior to removal. This is also compatible with PG production from guinea-pig endometrium in culture for 24h presented in section 3.1, where  $\text{PGF}_{2\alpha}$  production is high from day 15 and low from day 7 tissue, reflecting the in vivo state at the time of removal.

It is conceivable that the newly synthesised secreted proteins measured are cellular proteins released into the medium by cell lysis. However, Strinden and Shapiro (1983) showed that the majority of proteins secreted from the human proliferative endometrium are glycoproteins, which are not like the cellular proteins synthesised. Komm, Rusling and Lyttle (1986) investigating immature rat uterus in organ culture also showed that proteins, which were newly synthesised and then released into the medium, were different from newly synthesised cellular proteins. This indicates cell lysis is not a major contributory factor in the release of radiolabelled proteins into the culture medium.

There were no differences in the rates of cellular protein synthesis between day 7 and day 15 endometrium in culture but there was a greater rate of synthesis of secreted proteins from day 15 when compared to day 7 endometrium in culture. However, this technique is unable to distinguish between an increase in the general rate of synthesis of all secreted proteins and an increase in the production of specific secreted proteins. Furthermore, synthesis of specific proteins may be stimulated or inhibited at different stages of the cycle which may not change the measured rate of overall protein synthesis, but the actual proteins being synthesised would be very different. Proteins synthesised by the endometrium during the menstrual cycle of the human (Bell, Patel, Kirwan and Drife, 1986) and the oestrous cycle of the cow (Bartol, Roberts, Bazer and Thatcher, 1985) vary at different stages of the cycle.

The results presented in this study show that de novo protein synthesis occurred in the guinea-pig endometrium and that this preparation could be maintained for 24h in culture to measure total cellular and secreted protein synthesis. There was no difference in the total amounts of cellular proteins synthesised by day 7 and day 15 culture endometrium, but more secreted proteins were synthesised by day 15 cultured endometrium.

### 3.5 THE ROLE OF PROTEIN SYNTHESIS IN THE CONTROL OF PG PRODUCTION BY THE GUINEA-PIG UTERUS

#### INTRODUCTION

Oestradiol acting on a progesterone-primed uterus is probably the physiological stimulus that causes the relatively specific increase in  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus towards the end of the oestrous cycle (Poyser, 1983b). Many of the actions of oestradiol are mediated by an increase in protein synthesis (Brenner and West, 1975). Previous studies in the sheep (French and Casida, 1973) and in the guinea-pig (Poyser, 1979) have shown that the intra-uterine administration of actinomycin D, an inhibitor of DNA-dependent RNA synthesis, increases oestrous cycle length. In the study in the guinea-pig, Poyser (1979) showed that the in vitro  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  synthesizing capacities of homogenates of the uterus were reduced by intra-uterine actinomycin D treatment. This was reflected in vivo by increased peripheral plasma progesterone concentrations, presumably due to an inhibition of the production of the luteolytic factor ( $\text{PGF}_{2\alpha}$ ), therefore prolonging luteal function.

Recently, there has been much interest shown in the production of proteins both by the uterus and by the conceptus in early pregnancy, and the ability of these proteins to control PG synthesis. The lipocortins,  $\text{PLA}_2$  inhibitory proteins, have been shown to be produced by the human endometrium in culture (Gurpide, Markiewicz, Schatz and Hirata, 1986). Proteins with an inhibitory action on prostaglandin production have been shown to be produced by the embryo in early pregnancy in the cow (Knickerbocker, Thatcher, Bazer, Drost, Barron, Fincher and Roberts, 1986) and in the sheep (Fincher, Bazer, Hansen, Thatcher and Roberts, 1986).

Inhibitors of protein and RNA synthesis have been used to



investigate whether protein and RNA synthesis are necessary for a variety of physiological functions. If an inhibitor of protein or RNA synthesis alters such a function, it is inferred that the production of a protein(s) is required in controlling this physiological function, and other metabolic functions are not affected (Fagan and Goldberg, 1986). It is possible, therefore, that a protein is the mediator in the action of oestradiol in the stimulation of PGF<sub>2α</sub> synthesis.

The next series of experiments have examined the role of protein synthesis in the control of PG synthesis in the guinea-pig uterus. The effects of the in vivo and in vitro treatment with protein and RNA synthesis inhibitors on PG outputs from, PG synthesising capacities of, and protein synthesis by cultured and superfused, guinea-pig uterine tissues have been investigated. The aims of this series of experiments are:-

- (a) to investigate whether protein synthesis is inhibited in the endometrium by inhibitors of protein- and RNA-synthesis by measuring the incorporation of [<sup>3</sup>H]-leucine into proteins synthesised by the endometrium in culture.
- (b) to investigate the actions of protein and RNA synthesis inhibitors on PG outputs from and synthesising capacities of day 7 and day 15 endometrium in culture.
- (c) to investigate whether protein and RNA synthesis inhibitors have a rapid (i.e. less than 1h) inhibitory effect on PG synthesis by the guinea-pig uterus. A rapid inhibitory effect on PG production has been previously reported in skeletal muscle preparations (Turinsky, 1985; Fagan and Goldberg, 1986).
- (d) to investigate the effects of the intra-uterine injection of actinomycin D on day 10 of the oestrous cycle on PG production and

protein synthesis by the guinea-pig uterus on day 15 of the oestrous cycle.

### 3.5.1 THE EFFECTS OF PROTEIN AND RNA SYNTHESIS INHIBITORS ON PG PRODUCTION AND FRESH PROTEIN SYNTHESIS BY THE GUINEA-PIG ENDOMETRIUM IN CULTURE

#### INTRODUCTION

This experiment has investigated the role of actinomycin D (a DNA-dependent RNA synthesis inhibitor), cycloheximide (an inhibitor of the elongation step of protein transcription), and puromycin (an inhibitor of the synthesis of proteins by releasing nascent polypeptide chains before their synthesis is complete) on the PG outputs from and PG synthesising capacities (measured in homogenates) of cultured guinea-pig endometrium. The synthesis of cellular and secreted proteins has already been shown to occur in cultured guinea-pig endometrium in section 3.4. This section has also examined the actions of actinomycin D, cycloheximide and puromycin on this protein synthesis by endometrium in culture.

#### METHODS

Guinea-pigs on day 7 and day 15 of the oestrous cycle were killed and their uteri were removed in preparation for tissue culture, as described in section 2.2.1. Endometrium was dissected from the myometrium and small pieces were placed in Petri dishes as detailed in the tissue culture method (section 2.2.5). In Experiments 1 and 2, each dish contained 12-20mg endometrium and, in Experiment 3, additional dishes contained 30-60mg endometrium. The dishes were incubated in modified Kilner jars in the conditions described previously (section 2.2.5). All the tissue culture samples and ethyl

acetate extracts of the homogenate samples from Experiments 1 and 2 were stored at  $-20^{\circ}\text{C}$  before the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were measured by radioimmunoassay (section 2.3).

The details of each experiment are explained below. All protein synthesis inhibitors were administered in  $50\mu\text{l}$  sterile saline.

Experiment 1: Effects of protein synthesis inhibitors on PG outputs from guinea-pig endometrium cultures for 24h

The uteri from five day 7 and five day 15 guinea-pigs were removed and endometrium from each uterus was used to prepare eighteen Petri dishes for tissue culture (see above). The dishes were treated as follows: four dishes remained untreated (controls) and the remaining fourteen dishes were divided into seven pairs and these pairs were treated with one of the following concentrations of protein or RNA synthesis inhibitors; actinomycin D (1, 10 and  $50\mu\text{g/ml}$ ); cycloheximide (10 and  $50\mu\text{g/ml}$ ); puromycin (10 and  $50\mu\text{g/ml}$ ). These dishes were divided equally between two modified Kilner jars, so that there were two control dishes and one of each of the seven duplicate treatments in each jar (i.e. nine dishes/jar). These jars were gassed and incubated as specified above for 24h. The medium was removed every 6h, and the dishes were replenished with fresh medium and the same treatment. The dishes were replaced in the jars which were gassed and incubated as above.

Experiment 2: Effects of protein synthesis inhibitors on PG outputs from and PG synthesising capacities of guinea-pig endometrium in culture for up to 12h

The uteri from five day 7 and five day 15 guinea-pigs were removed and endometrium from each uterus was used to prepare sixteen Petri dishes for tissue culture (see above). These dishes were divided into four groups of four, and each group was treated as

follows. One group of dishes was left untreated (controls), and the remaining groups of dishes were treated with one of the following: actinomycin D ( $50\mu\text{g/ml}$ ); cycloheximide ( $10\mu\text{g/ml}$ ); puromycin ( $50\mu\text{g/ml}$ ). The dishes were divided equally between two modified Kilner jars, so each jar contained two dishes from each group (i.e. 8 dishes/jar), and the jars were gassed and incubated as specified above for up to 12h.

Immediately after the jars were placed in the incubator, two amounts of the remaining endometrium, each weighing approximately 12-20mg, were weighed accurately, homogenised and incubated for 60 min with  $2\mu\text{g}$  sodium arachidonate (prepared in  $10\mu\text{l}$  ethanol) as described previously (section 2.2.4.ii). These two homogenates measured the control PG synthesizing capacities of the endometrium at 0h. PGs were extracted from the incubates and were then redissolved in 5ml ethyl acetate as described in section 2.2.2.

After 6h of incubation, one control dish and one dish containing each treatment was removed from each jar (i.e. four dishes removed from each jar and eight dishes were therefore removed in total). The medium was harvested from the remaining dishes and replaced with fresh medium containing the same concentration of protein synthesis inhibitors. These dishes were replaced in the jars, which were gassed and incubated as before for a further 6h. After a total of 12h of incubation, these dishes were also removed.

Immediately after the dishes were removed from the jars at 6h and 12h, the endometrium was weighed, homogenised and incubated for 60 min with  $2\mu\text{g}$  sodium arachidonate as for the measurement of the 0h control endometrial PG synthesizing capacities as described above. The PGs were extracted and stored in 5ml ethyl acetate as above. These homogenates measured the PG synthesizing capacities of

endometrial homogenates after 6h and 12h of culture. The culture medium was collected for measurement of PG output.

### Experiment 3: Effects of protein synthesis inhibitors on endometrial protein synthesis

The uteri were removed from five day 7 and five day 15 guinea-pigs and endometrium from each uterus was placed in four Petri dishes for tissue culture (as explained above). Each dish contained 30-60mg endometrium and 10 $\mu$ Ci [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml) as specified in the procedure for measuring protein synthesis (section 2.4). The dishes were placed in a modified Kilner jar, which was gassed and incubated for 24h in the conditions given in section 2.2.5.

As soon as the jar was placed in the incubator, between 30 and 60mg of the remaining endometrium was placed in another Petri dish containing [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml) as in the method for the preparation for tissue culture as above. The tissue was washed with the [ $^3$ H]-leucine-containing culture medium in the dish. After 2 min incubation at room temperature, the endometrium was removed and weighed. The procedures detailing the measurement of [ $^3$ H]-leucine incorporation into cellular and secreted proteins by endometrial tissue were then followed, as detailed in section 2.4. The results from this dish measured the non-specific binding of [ $^3$ H]-leucine during the procedures used to measure [ $^3$ H]-leucine incorporation into cellular and secreted proteins.

The other dishes were removed from the jar after 24h of culture. The incorporation of [ $^3$ H]-leucine into cellular and secreted proteins was measured by analysing the endometrium and culture medium, respectively, as detailed in section 2.4.

### Statistical analysis and calculations

All statistical comparisons were made using Student's 't' test

(or a modified 't' test if the variances were unequal as shown by the variance ratio F-test). In Experiment 3, the amounts of [ $^3\text{H}$ ]-leucine which were bound non-specifically, and retained during the measurement of cellular and secreted proteins were subtracted from the values for the amounts of [ $^3\text{H}$ ]-leucine which were incorporated into the same type of protein synthesised during the 24h in culture. Also in Experiment 3, results were compared using the paired 't' test.

## RESULTS

### Experiment 1: Effects of protein synthesis inhibitors on PG outputs from guinea-pig endometrium cultured for 24h

The basal, control outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium cultured for 24h, with sampling every 6h, have been previously reported in detail in section 3.1 (see figure 3.1.b). The outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from day 7 guinea-pig endometrium in culture were not significantly changed by actinomycin D treatment (1, 10 and  $50\mu\text{g/ml}$ ) during the first 6h of culture or by actinomycin D ( $1\mu\text{g/ml}$ ) treatment throughout the culture period when compared to the respective control output value at the same time (Figure 3.5.1.a). The output of  $\text{PGF}_{2\alpha}$  was significantly ( $P < 0.05$ ) inhibited by 10 and  $50\mu\text{g}$  actinomycin D/ml treatment, during the three remaining sample periods between 6h and 24h of culture. The output of  $\text{PGE}_2$  was significantly ( $P < 0.05$ ) inhibited by actinomycin D ( $50\mu\text{g/ml}$ ) treatment between 6h and 12h of culture, and by actinomycin D (10 and  $50\mu\text{g/ml}$ ) treatment between 12h and 18h, and 18h and 24h of culture (Figure 3.5.1.a). Actinomycin D treatment exhibited no significant effect on 6-keto- $\text{PGF}_{1\alpha}$  output during 24h of culture of day 7 guinea-pig endometrium (Figure 3.5.1.a).

The output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium cultured for 24h was

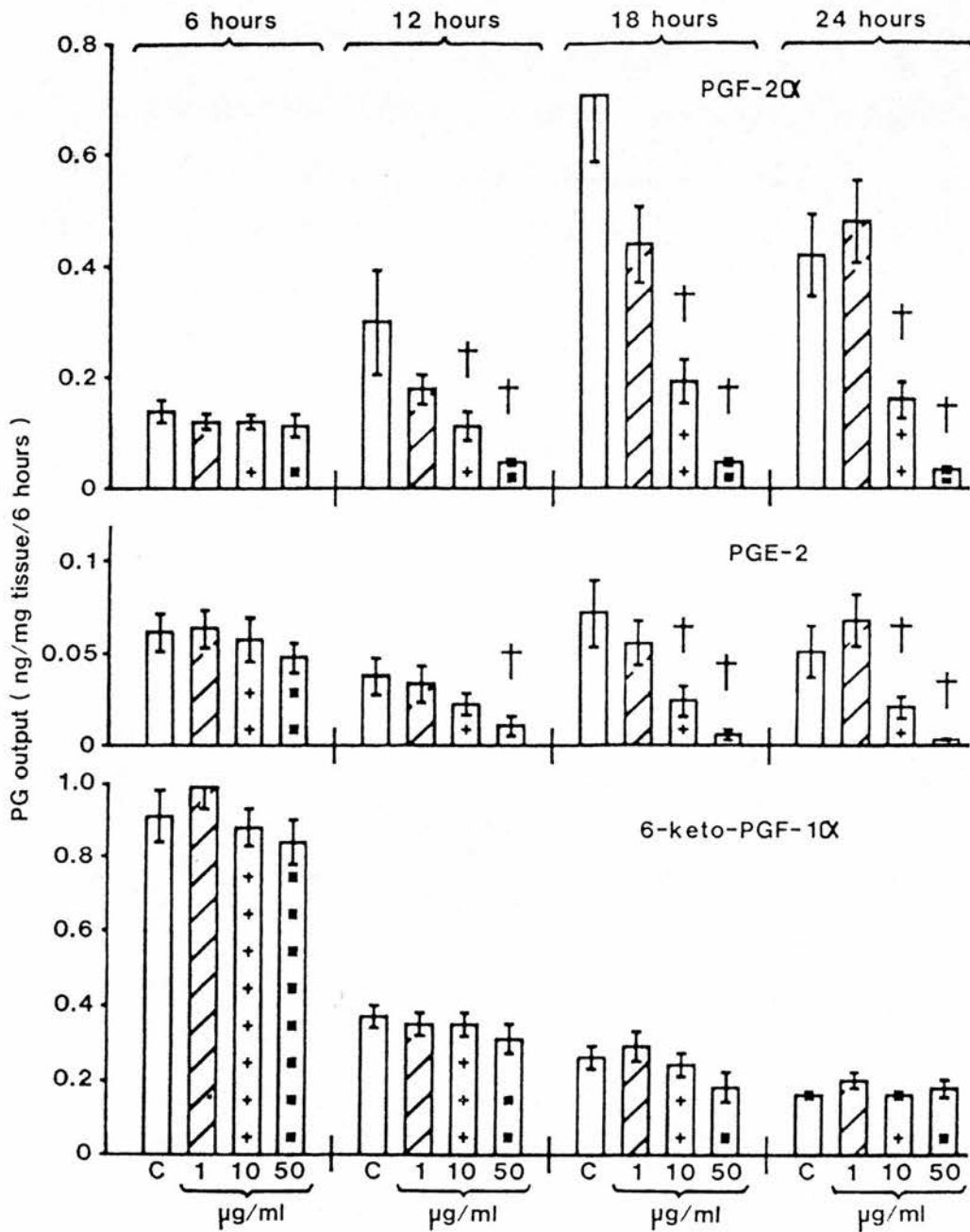


Figure 3.5.1.a

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with  $1\mu\text{g/ml}$  (hatched columns),  $10\mu\text{g/ml}$  (crossed columns) and  $50\mu\text{g/ml}$  (squared columns) actinomycin D.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG during the same time period.



not significantly changed by actinomycin D (1 and 10 $\mu$ g/ml) treatment throughout the culture period, except for 10 $\mu$ g actinomycin D/ml, treatment which significantly ( $P < 0.05$ ) inhibited PGF<sub>2 $\alpha$</sub>  output between 18h and 24h of culture (Figure 3.5.1.b). Actinomycin D (50 $\mu$ g/ml) treatment significantly ( $P < 0.05$ ) inhibited PGF<sub>2 $\alpha$</sub>  output from day 15 endometrium during all four sample periods between 0 and 24h of culture. The output of PGE<sub>2</sub> from day 15 endometrium was not significantly altered by actinomycin D (1 and 10 $\mu$ g/ml) treatment, but PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) inhibited by 50 $\mu$ g actinomycin D/ml treatment during all four sample periods in the 24h period of culture (Figure 3.5.1.b). Actinomycin D (1 and 10 $\mu$ g/ml) treatment had no significant effect on 6-keto-PGF<sub>1 $\alpha$</sub>  output throughout the 24h of culture, except for 10 $\mu$ g actinomycin D/ml treatment which significantly ( $P < 0.05$ ) inhibited 6-keto-PGF<sub>1 $\alpha$</sub>  output from day 15 endometrium between 12h and 18h of culture (Figure 3.5.1.b). Actinomycin D (50 $\mu$ g/ml) treatment significantly ( $P < 0.05$ ) inhibited 6-keto-PGF<sub>1 $\alpha$</sub>  output between 0 and 6h, 6h and 12h and 12h and 18h of culture (Figure 3.5.1.b).

Cycloheximide (10 and 50 $\mu$ g/ml) treatment had no significant effect on PGF<sub>2 $\alpha$</sub>  output from day 7 guinea-pig endometrium during the first 6h of culture (Figure 3.5.1.c). However, in the following three sample periods between 6h and 24h, cycloheximide treatment at both concentrations significantly ( $P < 0.05$ ) inhibited the increase in PGF<sub>2 $\alpha$</sub>  output as exhibited by the control values, and indeed further inhibited PGF<sub>2 $\alpha$</sub>  output when compared to the 0-6h control PGF<sub>2 $\alpha$</sub>  output. Paradoxically the output of PGE<sub>2</sub> was significantly ( $P < 0.05$ ) stimulated by 50 $\mu$ g cycloheximide/ml treatment, but not by 10 $\mu$ g cycloheximide/ml treatment, during the first 6h of culture (Figure 3.5.1.c). Cycloheximide treatment (both concentrations) had no



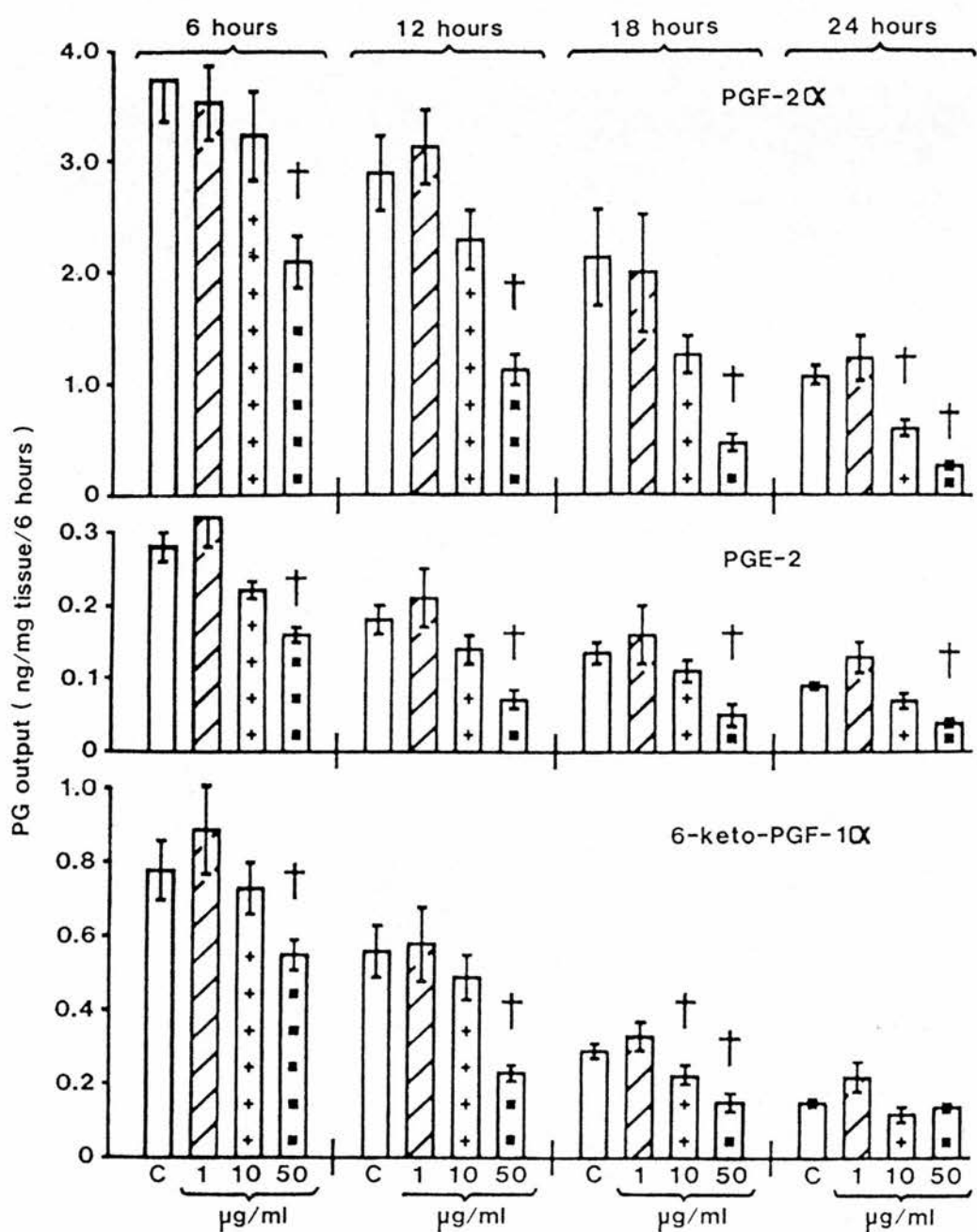


Figure 3.5.1.b

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 15 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with  $1\mu\text{g/ml}$  (hatched columns),  $10\mu\text{g/ml}$  (crossed columns) and  $50\mu\text{g/ml}$  (squared columns) actinomycin D.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG during the same time period.

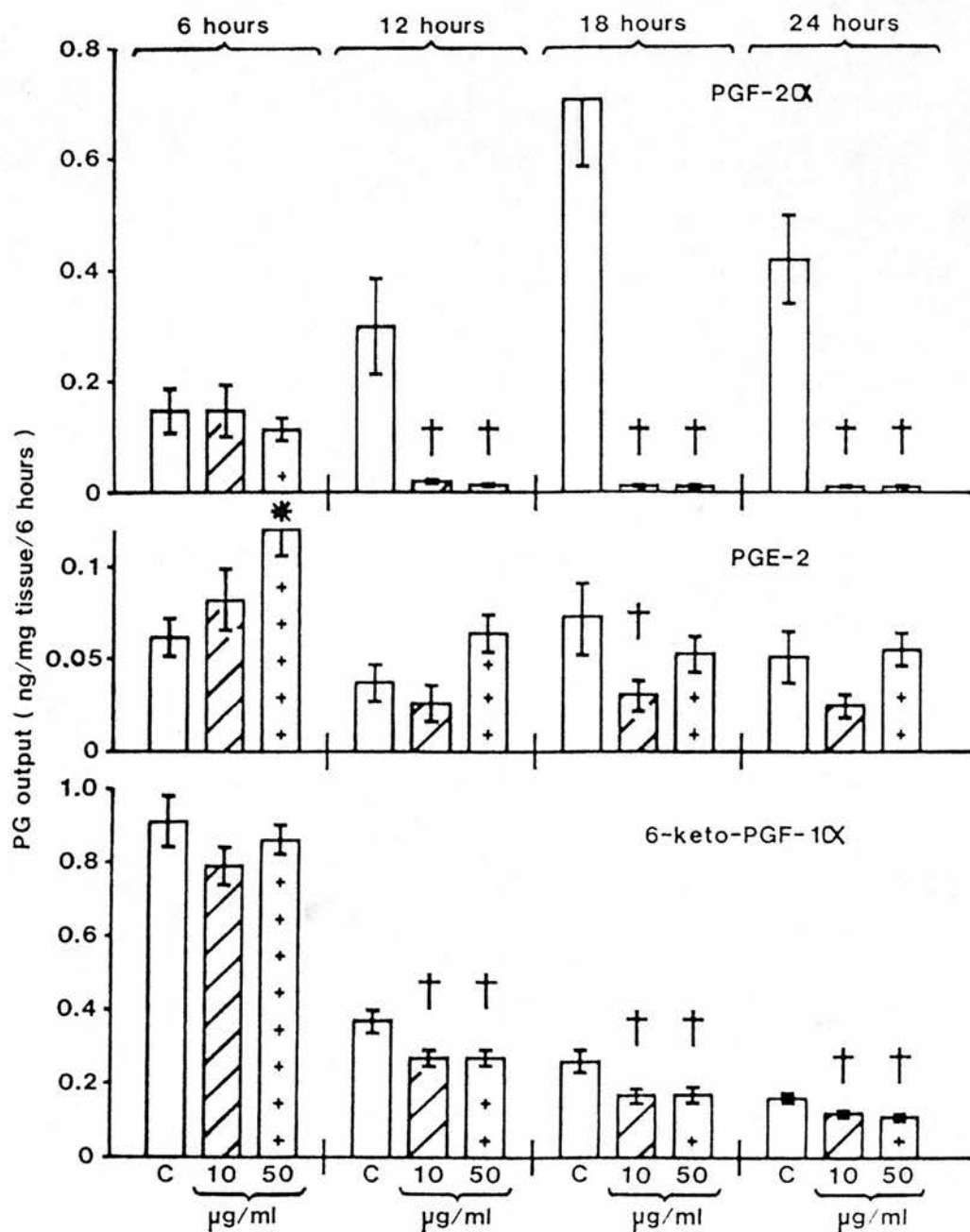


Figure 3.5.1.c

Mean (± s.e.m.; n = 10) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α from day 7 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with 10μg/ml (hatched columns) and 50μg/ml (crossed columns) cycloheximide.

† significantly (P < 0.05) lower than corresponding control value for the same PG during the same time period.

\* significantly (P < 0.05) higher than corresponding control value for the same PG during the same time period.

significant effect on PGE<sub>2</sub> output from day 7 endometrium during the remainder of the 24h period in culture, except 10µg cycloheximide/ml treatment significantly ( $P < 0.05$ ) inhibited PGE<sub>2</sub> output between 12h and 18h of culture. Treatment with cycloheximide (10 and 50µg/ml) had no significant action on 6-keto-PGF<sub>1α</sub> output during the first 6h of culture of day 7 endometrium (Figure 3.5.1.c). However, cycloheximide treatment at both concentrations significantly ( $P < 0.05$ ) reduced 6-keto-PGF<sub>1α</sub> output from day 7 endometrium between 6h and 12h, 12h and 18h and 18h and 24h of culture (Figure 3.5.1.c).

The output of PGF<sub>2α</sub> from day 15 endometrium was significantly ( $P < 0.05$ ) inhibited by cycloheximide (10 and 50µg/ml) treatment throughout the 24h period in culture (Figure 3.5.1.d). The outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 15 endometrium were not altered by cycloheximide treatment during the first 6h of culture (Figure 3.5.1.d). However, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs were significantly ( $P < 0.05$ ) inhibited by both concentrations of cycloheximide used between 6h and 12h, and 12h and 18h of culture. Cycloheximide treatment had no effect on PGE<sub>2</sub> output between 18h and 24h of culture, whereas cycloheximide (50µg/ml but not 10µg/ml) significantly ( $P < 0.05$ ) inhibited 6-keto-PGF<sub>1α</sub> output from day 15 endometrium between 18h and 24h of culture (Figure 3.5.1.d).

The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7 guinea-pig endometrium were not affected by puromycin (10 and 50µg/ml) treatment during the first 6h of culture (Figure 3.5.1.e). The outputs of PGF<sub>2α</sub> between 6h and 12h, 12h and 18h and 18h and 24h were significantly ( $P < 0.05$ ) lower than the respective control outputs of PGF<sub>2α</sub> at the same times, and were also significantly ( $P < 0.05$ ) lower than the control PGF<sub>2α</sub> output between 0 and 6h, following treatment with puromycin (both concentrations used). The output of PGE<sub>2</sub> from

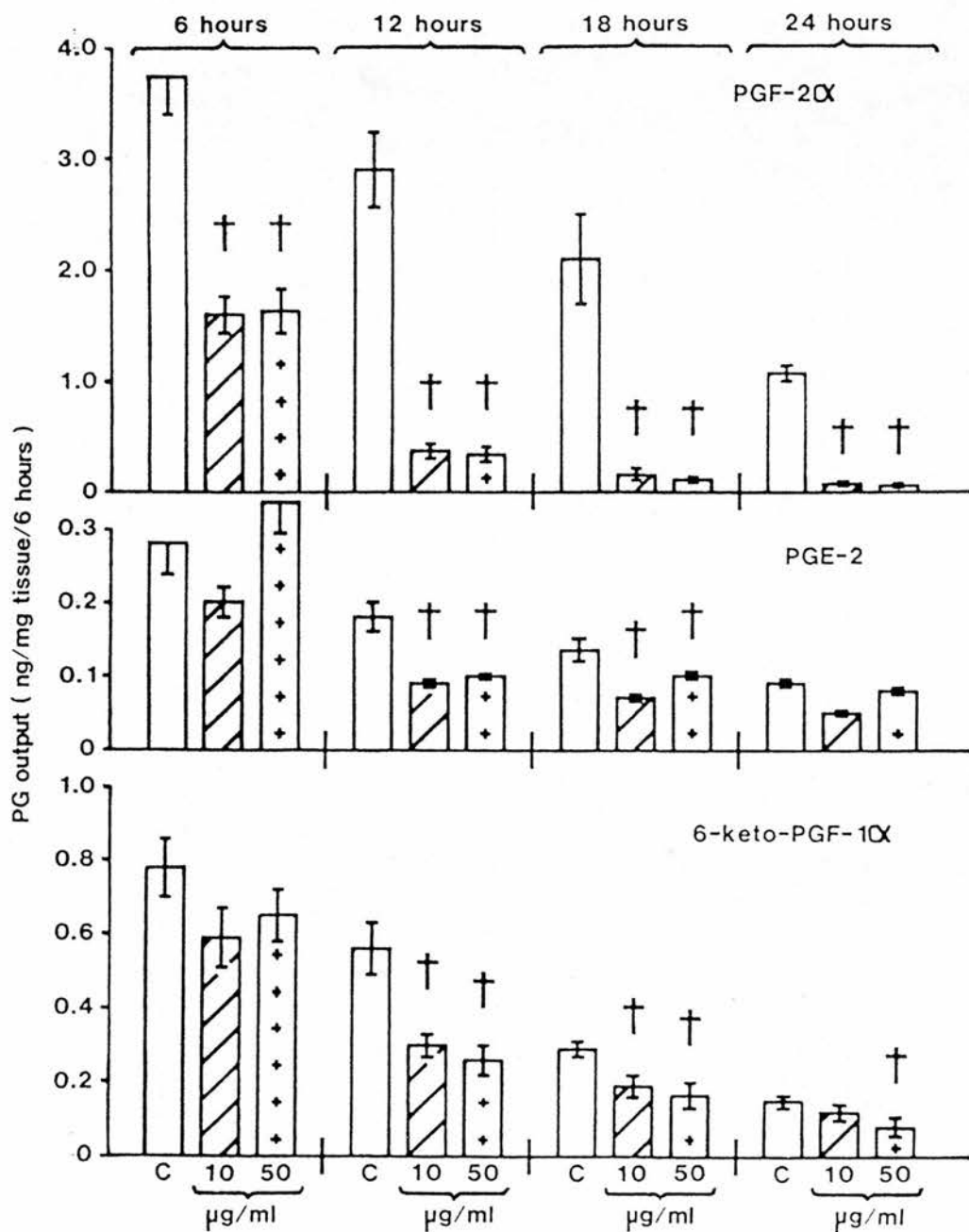


Figure 3.5.1.d

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 15 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with 10 $\mu\text{g/ml}$  (hatched columns) and 50 $\mu\text{g/ml}$  (crossed columns) cycloheximide.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG during the same time period.

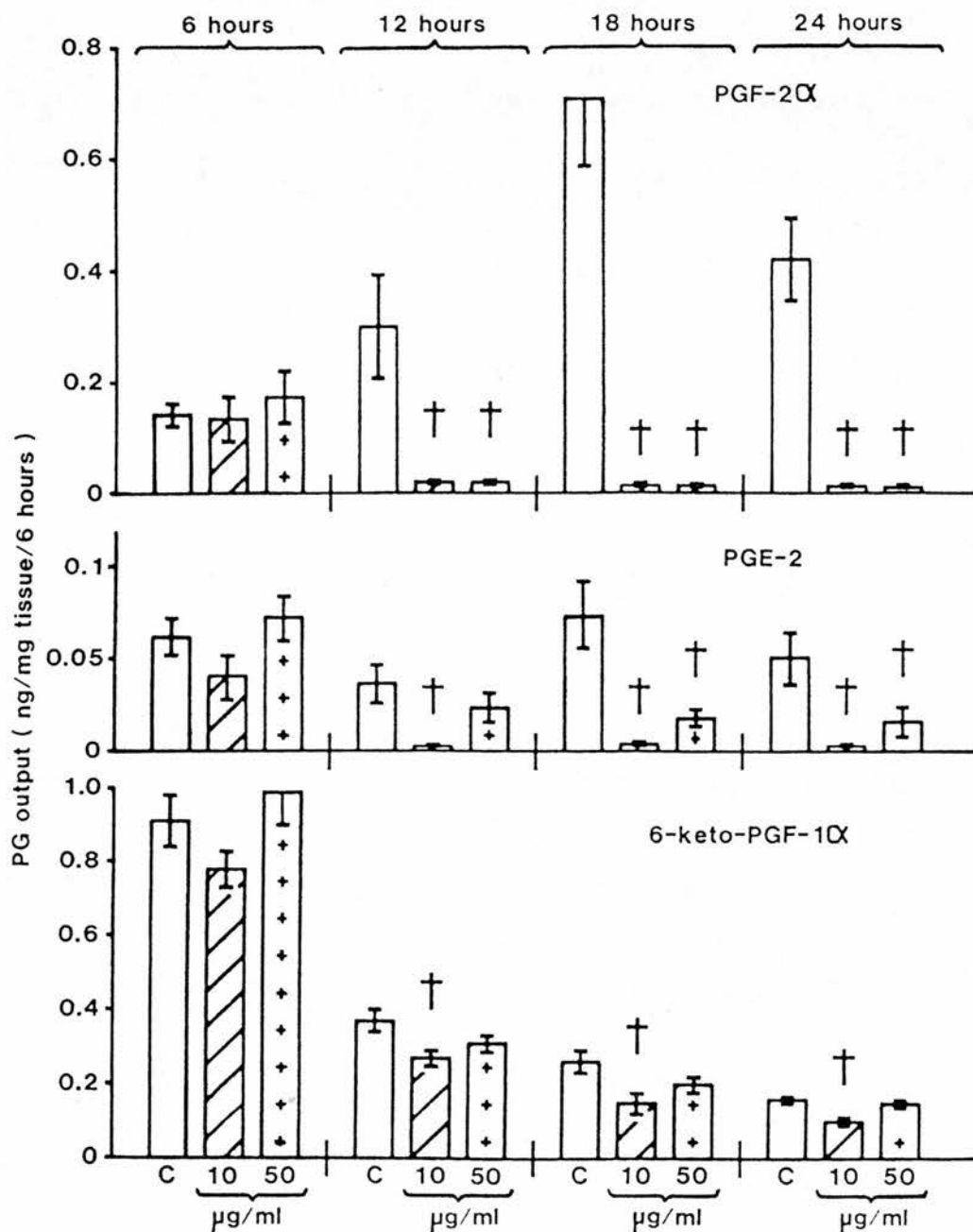


Figure 3.5.1.e

Mean (± s.e.m.; n = 10) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α from day 7 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with 10μg/ml (hatched columns) and 50μg/ml (crossed columns) puromycin.

† significantly (P < 0.05) lower than corresponding control value for the same PG during the same time period.

day 7 endometrium was significantly ( $P < 0.05$ ) inhibited by  $10\mu\text{g}$  puromycin/ml treatment between 6h and 12h of culture, and by 10 and  $50\mu\text{g}$  puromycin/ml treatment between 12h and 18h, and 18h and 24h of culture. Treatment with puromycin ( $10\mu\text{g}/\text{ml}$ ), but not puromycin ( $50\mu\text{g}/\text{ml}$ ) significantly ( $P < 0.05$ ) reduced 6-keto-PGF $_{1\alpha}$  output from day 7 endometrium during the three time periods between 6h and 24h of culture (Figure 3.5.1.e).

The output of PGF $_{2\alpha}$  from day 15 endometrium was significantly ( $P < 0.05$ ) inhibited by puromycin ( $50\mu\text{g}/\text{ml}$ ) treatment, but not by puromycin ( $10\mu\text{g}/\text{ml}$ ) treatment during the first 6h of culture (Figure 3.5.1.f). The outputs of PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 15 endometrium were not altered by puromycin treatment during the first 6h of culture (Figure 3.5.1.f). Puromycin (10 and  $50\mu\text{g}/\text{ml}$ ) treatment significantly ( $P < 0.05$ ) inhibited the outputs of PGF $_{2\alpha}$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 15 guinea-pig endometrium during the three periods of culture between 6h and 24h (Figure 3.5.1.f).

Experiment 2: Effects of protein synthesis inhibitors on PG outputs from and PG synthesizing capacities of guinea-pig endometrium in culture for up to 12h

Day 7: The control (untreated) outputs of PGE $_2$  and 6-keto-PGF $_{1\alpha}$ , but not PGF $_{2\alpha}$ , from day 7 endometrium significantly ( $P < 0.05$ ) decreased from 0-6h to 6h-12h of culture (Figure 3.5.1.g). Treatment with actinomycin D ( $50\mu\text{g}/\text{ml}$ ) and puromycin ( $50\mu\text{g}/\text{ml}$ ) had no significant effect on PGF $_{2\alpha}$ , PGE $_2$  or 6-keto-PGF $_{1\alpha}$  outputs from day 7 endometrium during the first 6h of culture (Figure 3.5.1.g). However, cycloheximide ( $10\mu\text{g}/\text{ml}$ ) treatment significantly ( $P < 0.05$ ) inhibited PGF $_{2\alpha}$  output, but not PGE $_2$  or 6-keto-PGF $_{1\alpha}$  outputs, from day 7 endometrium between 0 and 6h of culture (Figure 3.5.1.g). Treatment with actinomycin D, cycloheximide and puromycin significantly ( $P <$

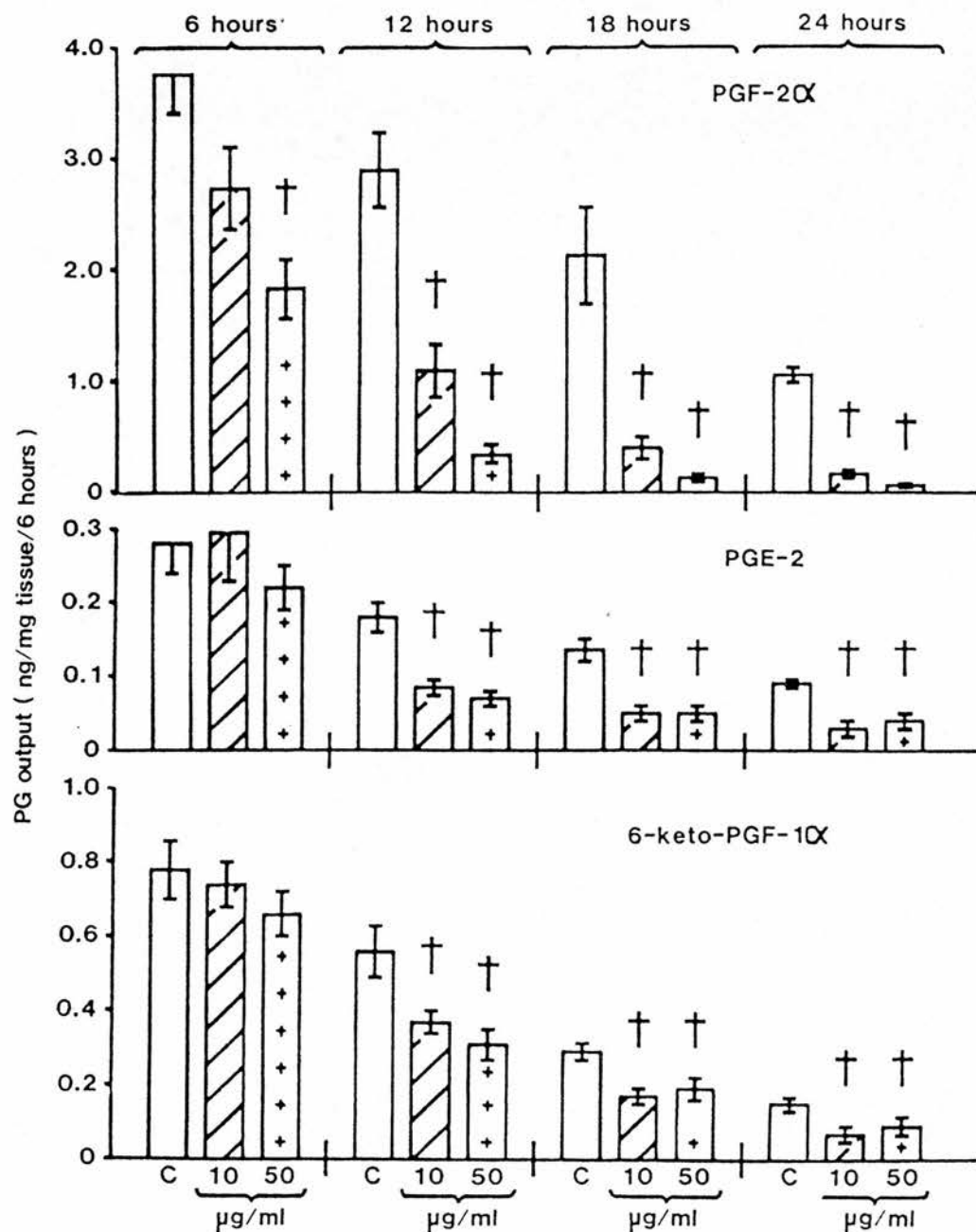


Figure 3.5.1.f

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 15 guinea-pig endometrium cultured for 24h (sampling every 6h) and not treated (control (C); open columns) or treated with 10 $\mu\text{g/ml}$  (hatched columns) and 50 $\mu\text{g/ml}$  (crossed columns) puromycin.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG during the same time period.

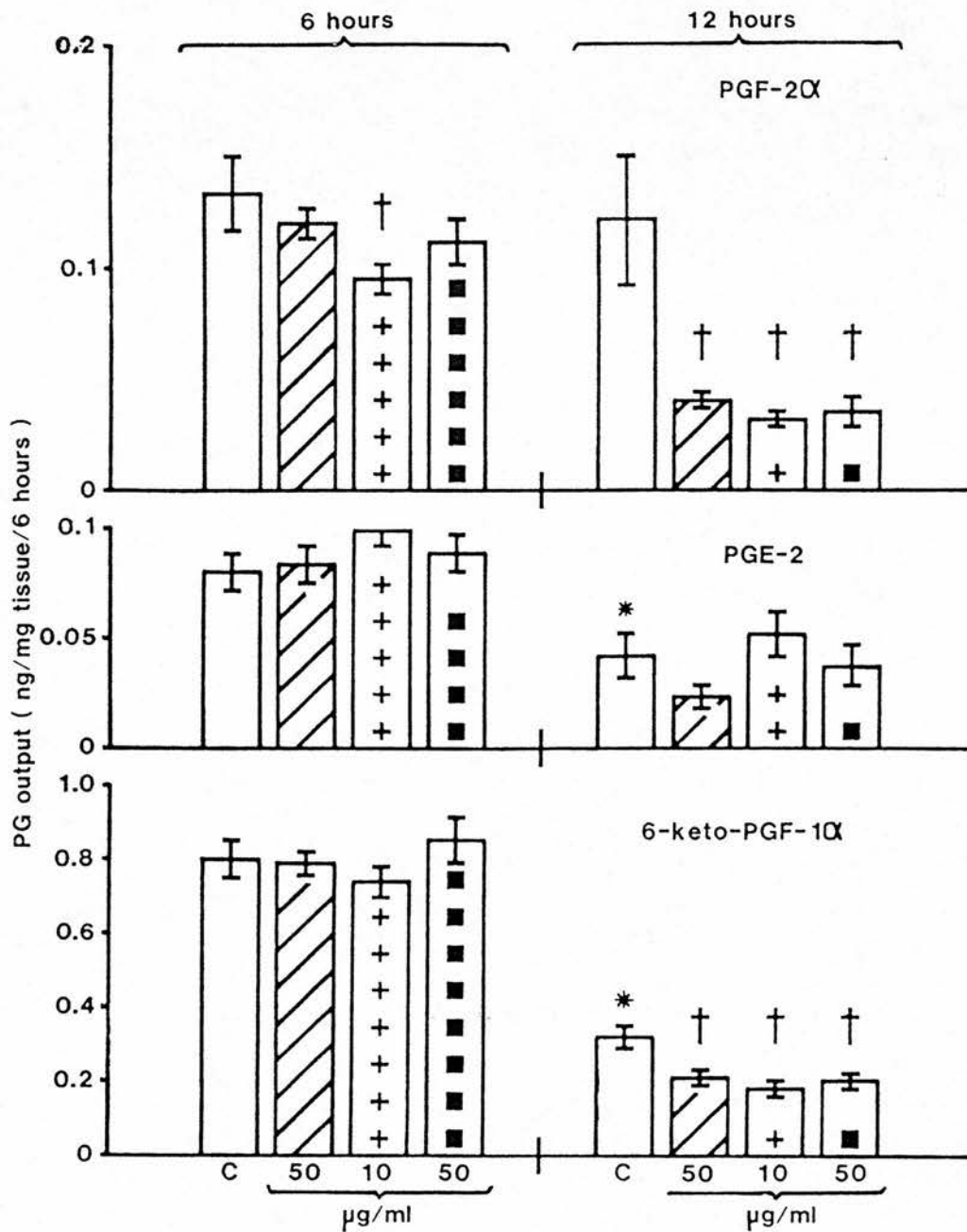


Figure 3.5.1.g

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 7 guinea-pig endometrium cultured for 12h (sampling every 6h) and not treated (control (C); open columns) or treated with actinomycin D ( $50\mu\text{g/ml}$ ; hatched columns), cycloheximide ( $10\mu\text{g/ml}$ ; crossed columns) and puromycin ( $50\mu\text{g/ml}$ ; squared columns).

† significantly ( $P < 0.05$ ) lower than corresponding control output value for the same PG during the same time period.

\* significantly ( $P < 0.05$ ) lower than 0-6h control output value for the same PG.



0.05) inhibited  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs, but not  $\text{PGE}_2$  output from day 7 endometrium in culture between 6h and 12h (Figure 3.5.1.g).

The  $\text{PGF}_{2\alpha}$  synthesizing capacity of untreated (control) day 7 endometrial homogenates was significantly ( $P < 0.05$ ) higher than the 0h control value after 12h, but not after 6h, of culture (Figure 3.5.1.h). The amount of 6-keto- $\text{PGF}_{1\alpha}$  synthesised by control day 7 endometrial homogenates was significantly ( $P < 0.05$ ) higher after both 6h and 12h of culture, and the amount of  $\text{PGE}_2$  synthesised was significantly ( $P < 0.05$ ) higher after 12h, when compared to the corresponding 0h control value (Figure 3.5.1.h). Actinomycin D, cycloheximide and puromycin treatment significantly ( $P < 0.05$ ) inhibited the synthesizing capacities of day 7 endometrial homogenates of all three PGs after 6h and 12h of culture when compared to their corresponding control value at the same time, except actinomycin D treatment had no significant inhibitory effect on the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity after 12h of culture. Actinomycin D treatment prevented the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity from the 0h control value after 12h of culture of day 7 endometrial homogenates (Figure 3.5.1.h). Cycloheximide and puromycin treatment not only prevented the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity of day 7 endometrial homogenates observed in control values after 12h of culture, but also further significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  synthesising capacity after 12h of culture when compared to the 0h control value. Actinomycin D and puromycin treatment prevented the increase in  $\text{PGE}_2$  synthesizing capacity of day 7 endometrium after 12h of culture, whereas treatment with cycloheximide not only prevented this increase in  $\text{PGE}_2$  synthesizing capacity, but also significantly ( $P < 0.05$ ) inhibited  $\text{PGE}_2$  synthesizing capacity after 12h of culture when compared to the 0h control value. All three protein or RNA synthesis

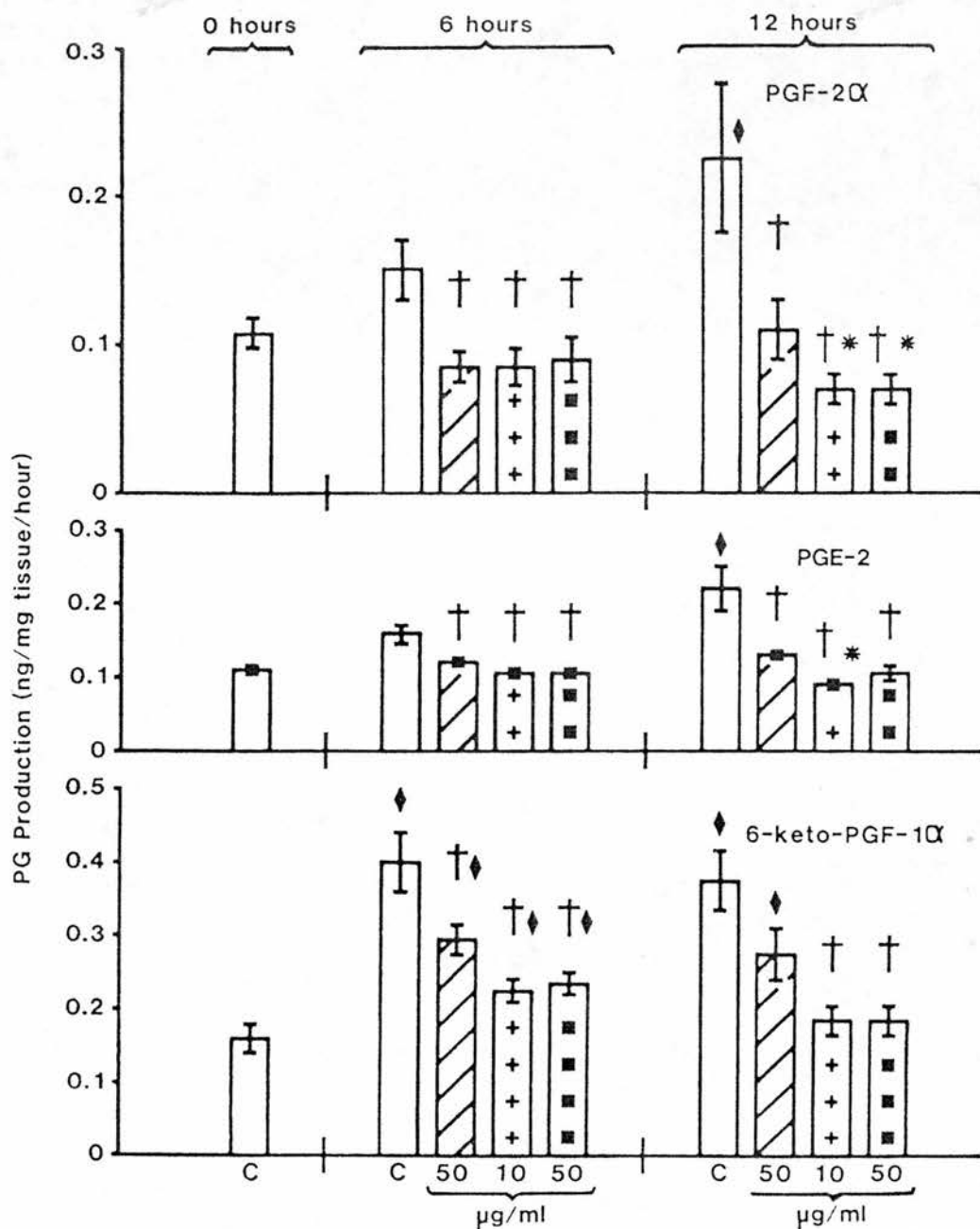


Figure 3.5.1.h

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  synthesized by homogenates of day 7 guinea-pig endometrium either not cultured and homogenised immediately (0h) or cultured for 6h or 12h before homogenisation and either not treated (control (C); open columns) or treated with actinomycin D (50 $\mu$ g/ml; hatched columns), cycloheximide (10 $\mu$ g/ml; crossed columns) and puromycin (50 $\mu$ g/ml; squared columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG production at the same time for the same PG.

♦ significantly ( $P < 0.05$ ) higher than the 0h control (C) PG production for the same PG.

\* significantly ( $P < 0.05$ ) lower than the 0h control (C) PG production for the same PG.

inhibitors used inhibited the 6-keto-PGF<sub>1α</sub> synthesizing capacity of day 7 endometrium after 6h of culture, but the synthesizing capacities were still significantly ( $P < 0.05$ ) higher than the 0h control value. Actinomycin D treatment significantly ( $P < 0.05$ ) inhibited the 6-keto-PGF<sub>1α</sub> synthesizing capacity of day 7 endometrium after 12h of culture although this synthesising capacity was still significantly ( $P < 0.05$ ) higher than the 0h control value. However, puromycin and cycloheximide treatment inhibited the 6-keto-PGF<sub>1α</sub> synthesizing capacity of day 7 endometrium homogenised after 12h of culture to values that were not significantly different from the 0h control value (Figure 3.5.1.h).

Day 15: The control outputs of 6-keto-PGF<sub>1α</sub>, but not the control outputs of PGF<sub>2α</sub> or PGE<sub>2</sub> from cultured day 15 endometrium declined significantly ( $P < 0.05$ ) between the 0-6h and 6h-12h periods of culture (Figure 3.5.1.i). All three protein or RNA synthesis inhibitors significantly ( $P < 0.05$ ) reduced PGF<sub>2α</sub> output from day 15 endometrium during both 6h culture periods (i.e. between 0 and 6h and 6h and 12h) compared to the control PGF<sub>2α</sub> output values during the same periods (Figure 3.5.1.i). Puromycin treatment, but not actinomycin D or cycloheximide treatment significantly ( $P < 0.05$ ) inhibited PGE<sub>2</sub> output from day 15 endometrium during the first culture period, and all three protein or RNA synthesis inhibitors significantly ( $P < 0.05$ ) reduced PGE<sub>2</sub> output between 6h and 12h of culture when compared to the control PGE<sub>2</sub> output value recorded during this period (Figure 3.5.1.i). The output of 6-keto-PGF<sub>1α</sub> from day 15 cultured endometrium was significantly ( $P < 0.05$ ) inhibited by cycloheximide, but not by actinomycin D or puromycin during the first 6h period of culture, and was significantly ( $P < 0.05$ ) inhibited by cycloheximide and puromycin, but not by actinomycin D, during the

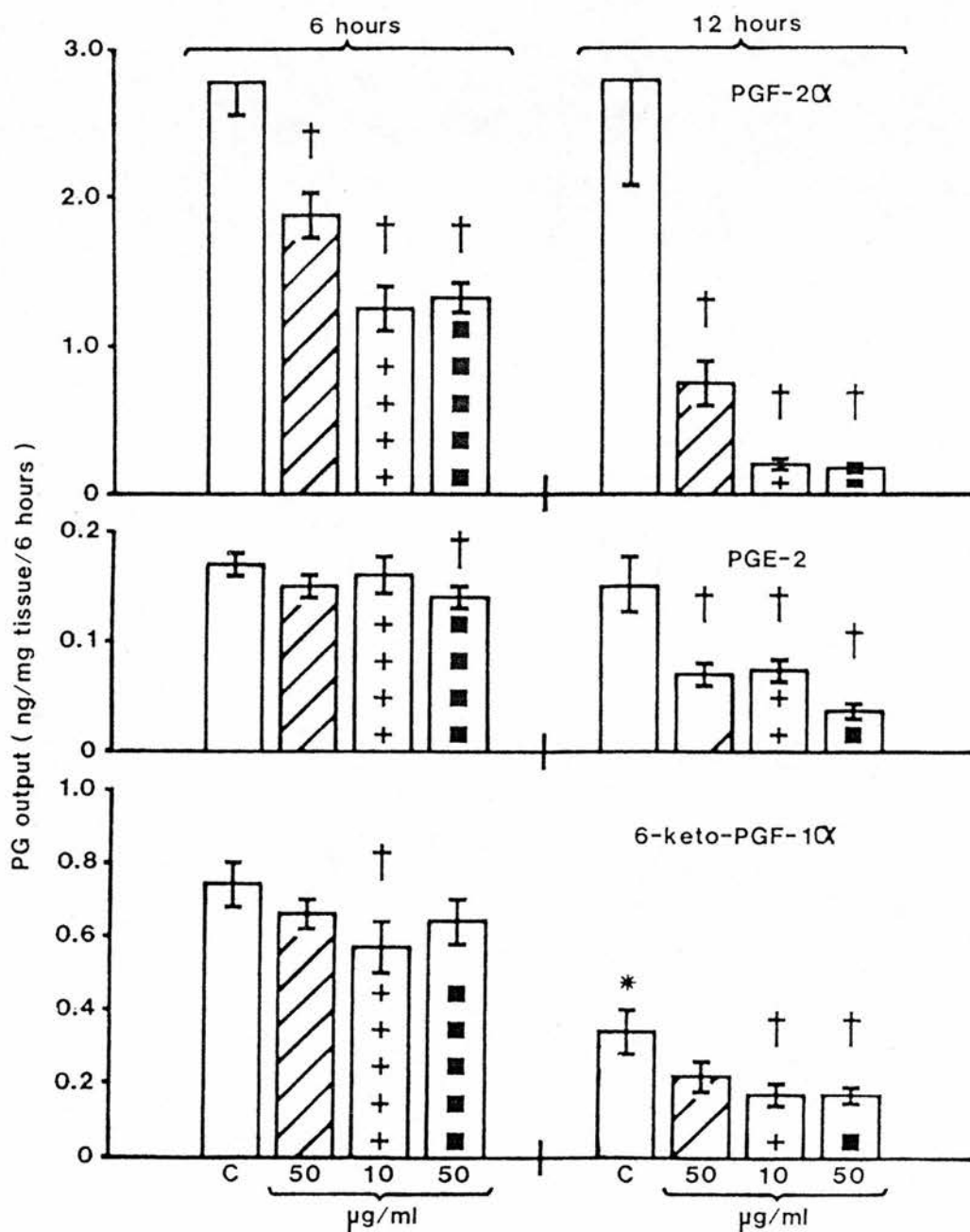


Figure 3.5.1.i

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 15 guinea-pig endometrium cultured for 12h (sampling every 6h) and not treated (control (C); open columns) or treated with actinomycin D ( $50\mu\text{g/ml}$ ; hatched columns), cycloheximide ( $10\mu\text{g/ml}$ ; crossed columns) and puromycin ( $50\mu\text{g/ml}$ ; squared columns).

† significantly ( $P < 0.05$ ) lower than corresponding control output value for the same PG during the same time period.

\* significantly ( $P < 0.05$ ) lower than 0-6h control output value for the same PG.

second 6h period of culture compared to the corresponding control PG output value at the same time (Figure 3.5.1.i.).

The synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  of untreated (control) day 15 endometrium after 6h of culture were significantly ( $P < 0.05$ ) higher than their respective 0h control values (Figure 3.5.1.j). This significantly ( $P < 0.05$ ) higher level compared to 0h synthesizing capacity values was maintained after 12h of culture for all three PGs. The day 15 endometrial synthesizing capacities of all three PGs were significantly ( $P < 0.05$ ) reduced by treatment with actinomycin D, cycloheximide and puromycin after both 6h and 12h of culture, when compared to their corresponding control value at the same time, except that actinomycin D treatment did not significantly alter the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity after 6h of culture compared to the corresponding control value (Figure 3.5.1.j). After actinomycin D treatment both the  $\text{PGF}_{2\alpha}$  synthesizing capacity after 6h and the 6-keto- $\text{PGF}_{1\alpha}$  synthesising capacity after 6h and 12h were still significantly ( $P < 0.05$ ) higher than 0h control values. The  $\text{PGF}_{2\alpha}$  synthesizing capacity of day 15 endometrium treated with actinomycin D was reduced to a value after 12h of culture that was not different from the 0h control value. Treatment with cycloheximide prevented the increase in  $\text{PGF}_{2\alpha}$  synthesising capacity exhibited by control day 15 endometrium after 6h of culture, and further, significantly ( $P < 0.05$ ) reduced the  $\text{PGF}_{2\alpha}$  synthesizing capacity of day 15 endometrium cultured for 12h when compared to the 0h control value. The increase in control,  $\text{PGE}_2$  synthesizing capacity after 6h and 12h of culture was not only prevented but was further and significantly ( $P < 0.05$ ) inhibited following treatment with cycloheximide when compared to the 0h control value. The increases in the synthesizing capacities of all three PGs that occurred in

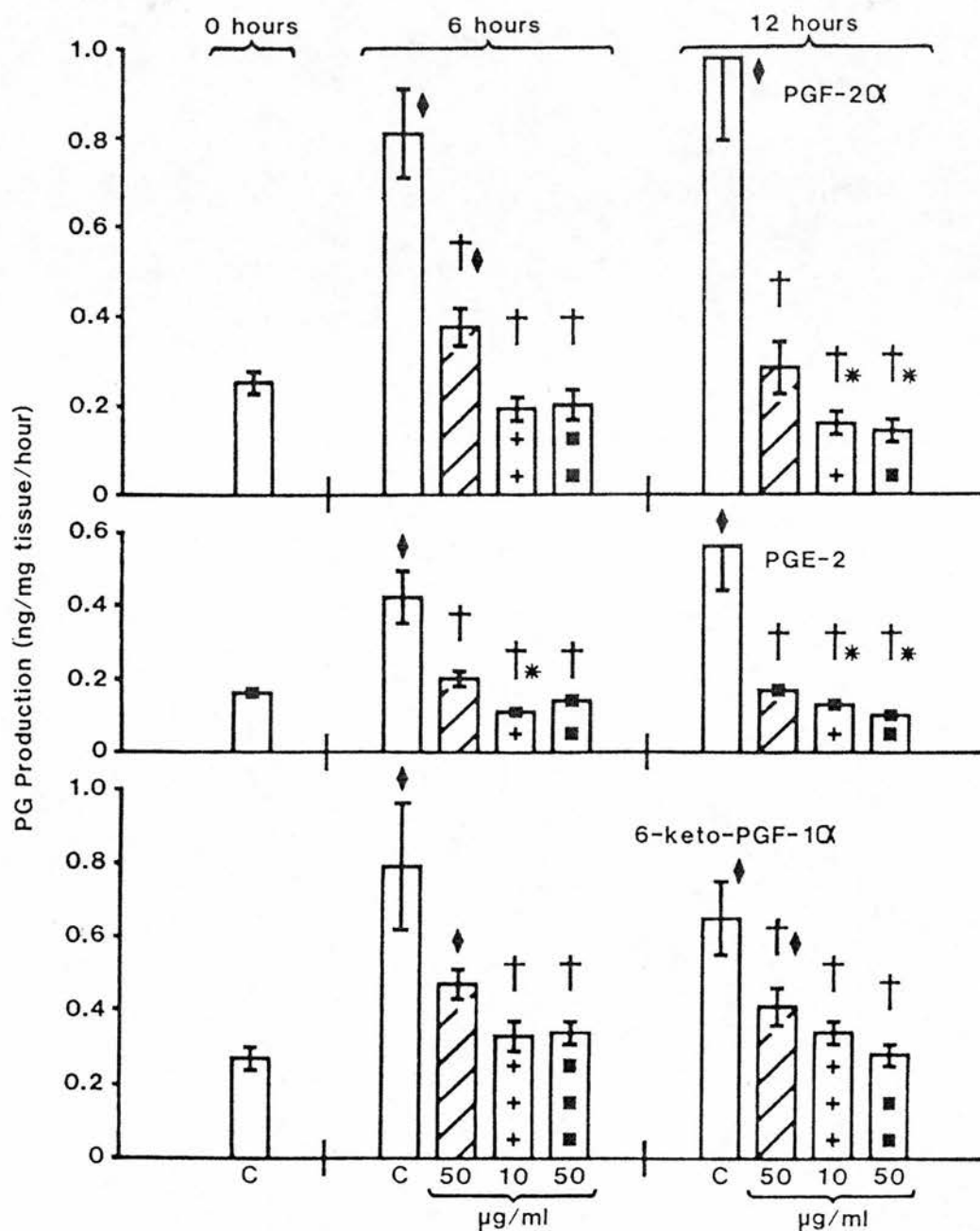


Figure 3.5.1.i

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesized by homogenates of day 15 guinea-pig endometrium either not cultured and homogenised immediately (0), or cultured for 6h or 12h before homogenisation and either not treated (control (C); open columns) or treated with actinomycin D ( $50\mu\text{g/ml}$ ; hatched columns), cycloheximide ( $10\mu\text{g/ml}$ ; crossed columns) and puromycin ( $50\mu\text{g/ml}$ ; squared columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG production at the same time for the same PG.

♦ significantly ( $P < 0.05$ ) higher than the 0h control (C) PG production for the same PG.

\* significantly ( $P < 0.05$ ) lower than the 0h control (C) PG production for the same PG.

control day 15 endometrium during 12h of culture were prevented by puromycin treatment after both 6h and 12h of culture. In addition, puromycin treatment further reduced the endometrial synthesizing capacities of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  to values which were significantly ( $P < 0.05$ ) lower than the 0h control value for the same PG (Figure 3.5.1.j).

### Experiment 3

Figure 3.5.1.k shows the incorporation of  $[^3\text{H}]$ -leucine into cellular and secreted proteins synthesised by day 7 and day 15 endometrium cultured for 24h, and not treated (control) or treated with protein and RNA synthesis inhibitors. In the control dishes there were significantly ( $P < 0.05$ ) greater amounts of  $[^3\text{H}]$ -leucine incorporated into secreted proteins synthesised by day 15 endometrium than by day 7 endometrium in culture for 24h. There was no significant difference between the amounts of  $[^3\text{H}]$ -leucine incorporated into the cellular proteins by day 7 and day 15 cultured endometrium. These results are in agreement with the results obtained for the incorporation of  $[^3\text{H}]$ -leucine into cellular and secreted proteins synthesised by day 7 and day 15 endometrium cultured for 24h and which are presented previously in section 3.4.

Treatment with actinomycin D ( $50\mu\text{g/ml}$ ), cycloheximide ( $10\mu\text{g/ml}$ ) and puromycin ( $50\mu\text{g/ml}$ ) significantly ( $P < 0.05$ ) inhibited the amounts of  $[^3\text{H}]$ -leucine incorporated into cellular and secreted proteins by day 7 and day 15 endometrium in culture for 24h (Figure 3.5.k).

### CONCLUSIONS

Actinomycin D, cycloheximide and puromycin had an inhibitory effect on the syntheses of cellular and secreted proteins by both day 7 and day 15 endometrium. Therefore, the concentrations of protein

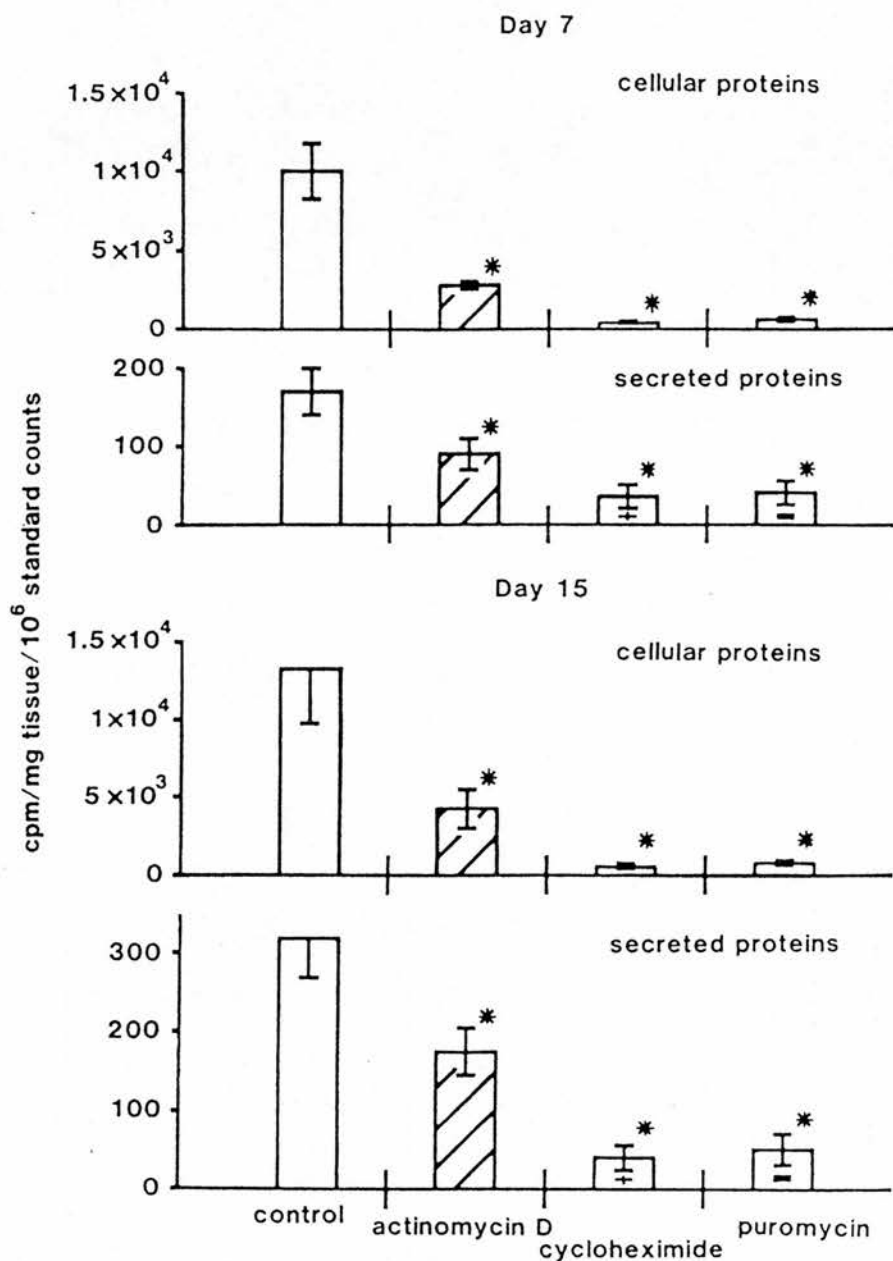


Figure 3.5.1.k

Mean ( $\pm$  s.e.m.;  $n = 5$ ) of  $[^3\text{H}]$ -leucine incorporation (measured as cpm/mg tissue/ $10^6$  standard counts) into cellular and secreted proteins synthesised by day 7 and day 15 guinea-pig endometrium in culture for 24h, not treated (controls; open columns) or treated with actinomycin D ( $50\mu\text{g/ml}$ ; hatched columns), cycloheximide ( $10\mu\text{g/ml}$ ; crossed columns) and puromycin ( $50\mu\text{g/ml}$ ; squared columns).

† significantly ( $P < 0.05$ ) higher than the control value for day 7 secreted proteins (by the paired 't' test).

\* significantly ( $P < 0.05$ ) lower than  $[^3\text{H}]$ -leucine incorporation into control proteins of the same type synthesised by endometrium from guinea-pigs on the same day of the cycle (by the paired 't' test).



and RNA synthesis inhibitors used are adequate to inhibit protein synthesis, and it may be inferred that any effects on PG synthesis are due to this inhibition of protein synthesis.

Treatment with actinomycin D (10 and 50  $\mu\text{g/ml}$ , but not 1  $\mu\text{g/ml}$ ) had an inhibitory effect on  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , but not 6-keto- $\text{PGF}_{1\alpha}$  outputs from cultured day 7 guinea-pig endometrium, but this inhibitory effect was not immediate since it only became apparent during the second (i.e. 6h to 12h) period of culture. Only the highest concentration of actinomycin D inhibited the outputs of all three PGs from cultured day 15 endometrium. However, inhibition by actinomycin D was apparent during the first (i.e. 0 to 6h) period of culture.

Cycloheximide treatment had a major inhibitory effect on  $\text{PGF}_{2\alpha}$  output, and a lesser inhibitory effect on  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from day 15 guinea-pig endometrium in culture. The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  exhibited a 6h delay before inhibition of output was observed. However, the output of  $\text{PGF}_{2\alpha}$  was inhibited during the first (i.e. 0 to 6h) period of culture.

Puromycin treatment had a major inhibitory action on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and, to a lesser extent, 6-keto- $\text{PGF}_{1\alpha}$  from both day 7 and day 15 cultured endometrium. The output of  $\text{PGF}_{2\alpha}$  was not inhibited by puromycin treatment of day 7 endometrium during the first 6h of culture, but puromycin (50  $\mu\text{g/ml}$ ) treatment of day 15 endometrium was inhibitory during the first 6h of culture. This inhibitory effect on the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , from both day 7 and day 15 endometrium was not present during the first (0 to 6h) culture period.

The endometrial synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were reduced by treatment with cycloheximide, puromycin

and, to a slightly lesser extent, actinomycin D. However, there were discrepancies between the inhibitory actions of protein or RNA synthesis inhibitors on the synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , after 6h of culture, and the measured output for the same PG during the first 6h of culture. For example, the synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  in homogenates of endometrium from both day 7 and day 15 guinea-pigs were inhibited after 6h of culture by actinomycin D, cycloheximide and puromycin (except actinomycin D treatment which had no inhibitory effect on the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 15 endometrium). However, none of these protein synthesis inhibitors had any effect on; (i) PG output from day 7 endometrium during the first 6h (i.e. 0 - 6h) of culture (except for cycloheximide which significantly inhibited  $\text{PGF}_{2\alpha}$  output in Experiment 2, and reduced  $\text{PGF}_{2\alpha}$  output, but not significantly in Experiment 1), (ii)  $\text{PGE}_2$  output from day 15 endometrium during the first 6h period of culture, except for puromycin (iii) 6-keto- $\text{PGF}_{1\alpha}$  output from day 15 endometrium except for cycloheximide. These discrepancies provide further evidence that the synthesizing capacity (and hence the concentration of PGH synthase) is not the rate-limiting step which controls PG synthesis by the guinea-pig endometrium. Previous evidence was presented in section 3.2 showing differences between PG outputs and PG synthesizing capacities. However, care must be taken in the interpretation of these results because most of the PGs released during the 0 - 6h period of culture may have been released early in this period of culture, whereas the respective synthesizing capacities may only be inhibited during the later stages of the culture period, to produce an observed decrease in PG synthesizing capacity. Therefore, the output and the PG synthesizing capacity may both decrease towards the end of the first

6h of culture, but this change in the output cannot be observed due to the high output occurring early in the period masking the effect. This means that this evidence does not necessarily preclude output being linked to PG synthesizing capacity (and hence PGH synthase concentration).

### 3.5.2 THE EFFECTS OF ACTINOMYCIN D, CYCLOHEXIMIDE AND PUROMYCIN ON PG OUTPUTS FROM AND PG SYNTHESIZING CAPACITIES OF GUINEA-PIG UTERUS SUPERFUSED IN VITRO

#### INTRODUCTION

Prostaglandin output from skeletal muscle has been shown to be very rapidly inhibited by protein and RNA synthesis inhibitors. In rat soleus muscle preparations, Turinsky and Loegering (1985) using cycloheximide, and Turinsky (1985) using puromycin and emitine showed that PGE<sub>2</sub> output is inhibited within 1h of treatment. Fagan and Goldberg (1986) using the rat extensor digitorum longus muscle preparation have shown a very rapid inhibitory effect (within 10 min) on PGE<sub>2</sub> and PGI<sub>2</sub> synthesis after treatment with cycloheximide, puromycin and actinomycin D. It has been suggested from these results that this inhibitory effect of protein and RNA synthesis inhibitors on PG output is due to a rapid inhibition of PGH synthase production, and that this enzyme exhibits a very rapid turnover of synthesis and breakdown so controlling PG production (Fagan and Goldberg, 1986).

The aims of this study were to investigate the short term actions (up to 1h) of the protein inhibitors cycloheximide and puromycin, and of the inhibitor of DNA-dependent RNA synthesis, actinomycin D, on the PG outputs from and PG synthesizing capacities of the guinea-pig uterus superfused in vitro.

## METHODS

Thirty guinea-pigs were used and each had exhibited at least two oestrous cycles of normal length prior to use. The animals were killed and the uteri removed in preparation for superfusion as described in section 2.2.1.

The uteri from five day 7 and five day 15 animals were divided into their two uterine horns. Each horn was weighed, then "opened" and prepared for superfusion as explained in section 2.2.3. Both uterine horns were superfused independently (5ml Krebs' solution/min) in the conditions previously detailed (section 2.2.3). Each horn was initially superfused for a "settling" period of 60 min, then superfusate samples were collected every 10 min for the next 80 min (i.e. 8 samples/uterine horn; samples 1 to 8). Between 90 and 110 min (i.e. samples 4 and 5) the normal Krebs' solution superfusing one horn (test horn; T) was replaced with Krebs' solution containing actinomycin D (50 $\mu$ g/ml). The other horn remained untreated and acted as a control (C). Following collection, the pH of each superfusate sample was lowered to 4.0 and the PGs were extracted and then redissolved in 10ml ethyl acetate (section 2.2.2). Immediately after the period of superfusion, the endometrium and the myometrium were separated by dissection, weighed accurately, homogenised and incubated for 60 min as explained in section 2.2.4.i. These homogenates measured the PG synthesizing capacities of their respective tissues. PGs were extracted from the incubates and redissolved in 5ml ethyl acetate (section 2.2.2). All ethyl acetate extracts of incubate and superfusate samples were stored at -20°C before the amounts of PGF<sub>2</sub> $\alpha$ , PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  present were measured by radioimmunoassay (section 2.3).

This procedure was repeated with uteri from five day 7 and five

day 15 guinea-pigs, except the Krebs' solution superfusing the test horns during samples 4 and 5 contained cycloheximide ( $50\mu\text{g/ml}$ ). The procedure was also repeated (as above) with puromycin ( $50\mu\text{g/ml}$ ) added to the Krebs' solution superfusing the test horn during samples 4 and 5.

### Statistical Tests

Duncan's multiple range test was used to test for significant changes in the output of a PG during the superfusion period. Student's 't'-test was used to compare PG synthesising capacities with their respective control values, for the same PG from the same tissue removed on the same day of the cycle.

### RESULTS

Actinomycin D treatment ( $50\mu\text{g/ml}$ ) significantly ( $P < 0.05$ ) stimulated  $\text{PGF}_{2\alpha}$  output from day 7 superfused uterine horns during the first 10 min (sample 4) of treatment (Figure 3.5.2.a). However, actinomycin D treatment had no significant effect on  $\text{PGF}_{2\alpha}$  output from day 15 superfused uterine horns (Figure 3.5.2.b).  $\text{PGE}_2$  output from day 7 superfused uterine horns was not significantly changed by actinomycin D treatment (Figure 3.5.2.a), whereas  $\text{PGE}_2$  output from day 15 superfused uterine horns was significantly ( $P < 0.05$ ) stimulated between 10 and 20 min (sample 5) of treatment with actinomycin D (Figure 3.5.2.b). The output of 6-keto- $\text{PGF}_{1\alpha}$  from both day 7 and day 15 superfused uterine horns was significantly ( $P < 0.05$ ) stimulated during the 20 min treatment period (samples 4 and 5) by actinomycin D (Figures 3.5.2.a and 3.5.2.b, respectively).

Cycloheximide treatment ( $50\mu\text{g/ml}$ ) significantly ( $P < 0.05$ ) stimulated  $\text{PGF}_{2\alpha}$  output from day 7 superfused uterine horns during samples 4 and 5 (Figure 3.5.2.c), but cycloheximide treatment

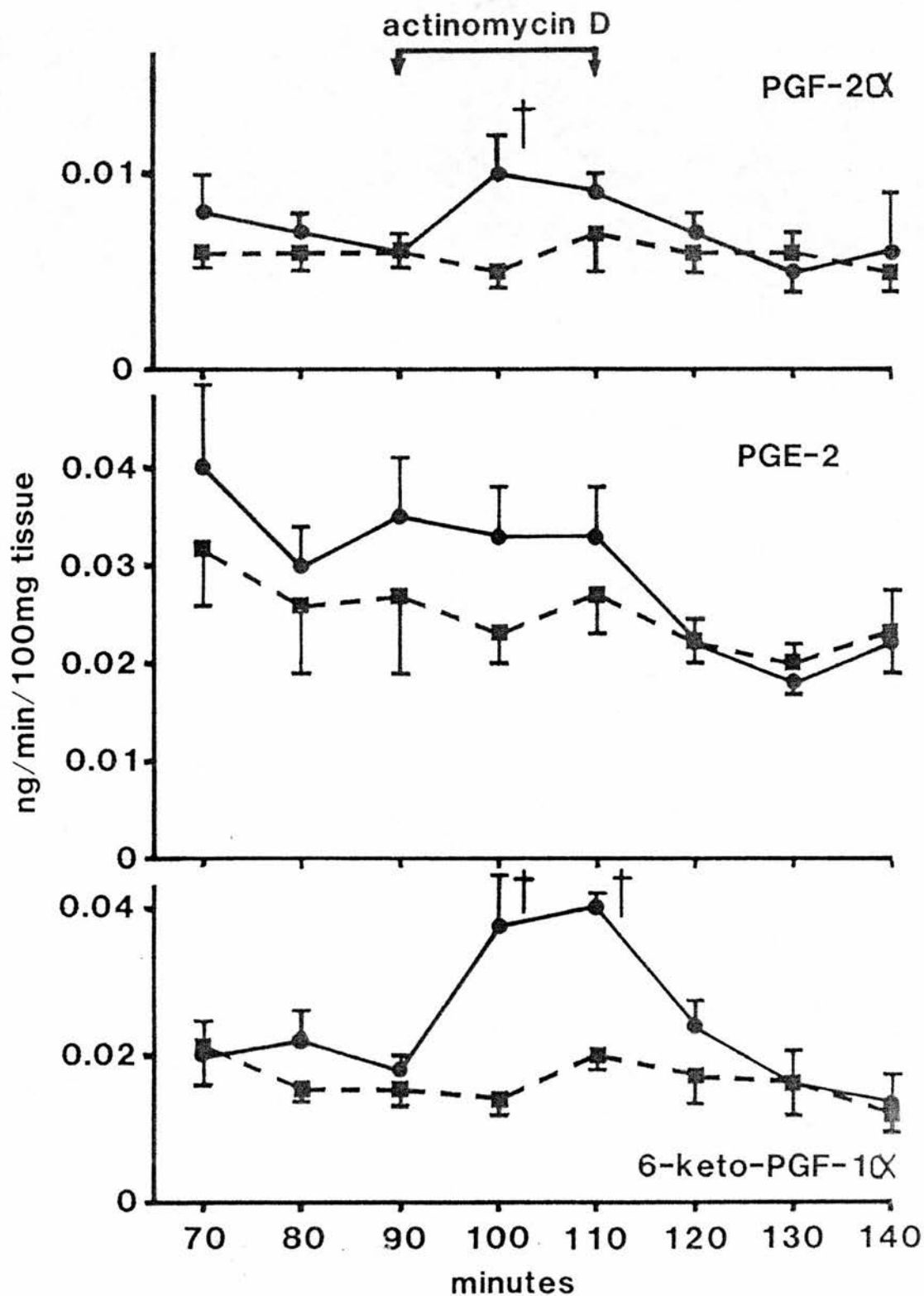


Figure 3.5.2.a

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of PGF $_{2\alpha}$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 7 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\square - \square$ ) or presence (treatment (T);  $\bullet - \bullet$ ) of actinomycin D (50 $\mu$ g/ml) between 90 min and 110 min.

$\dagger$  significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

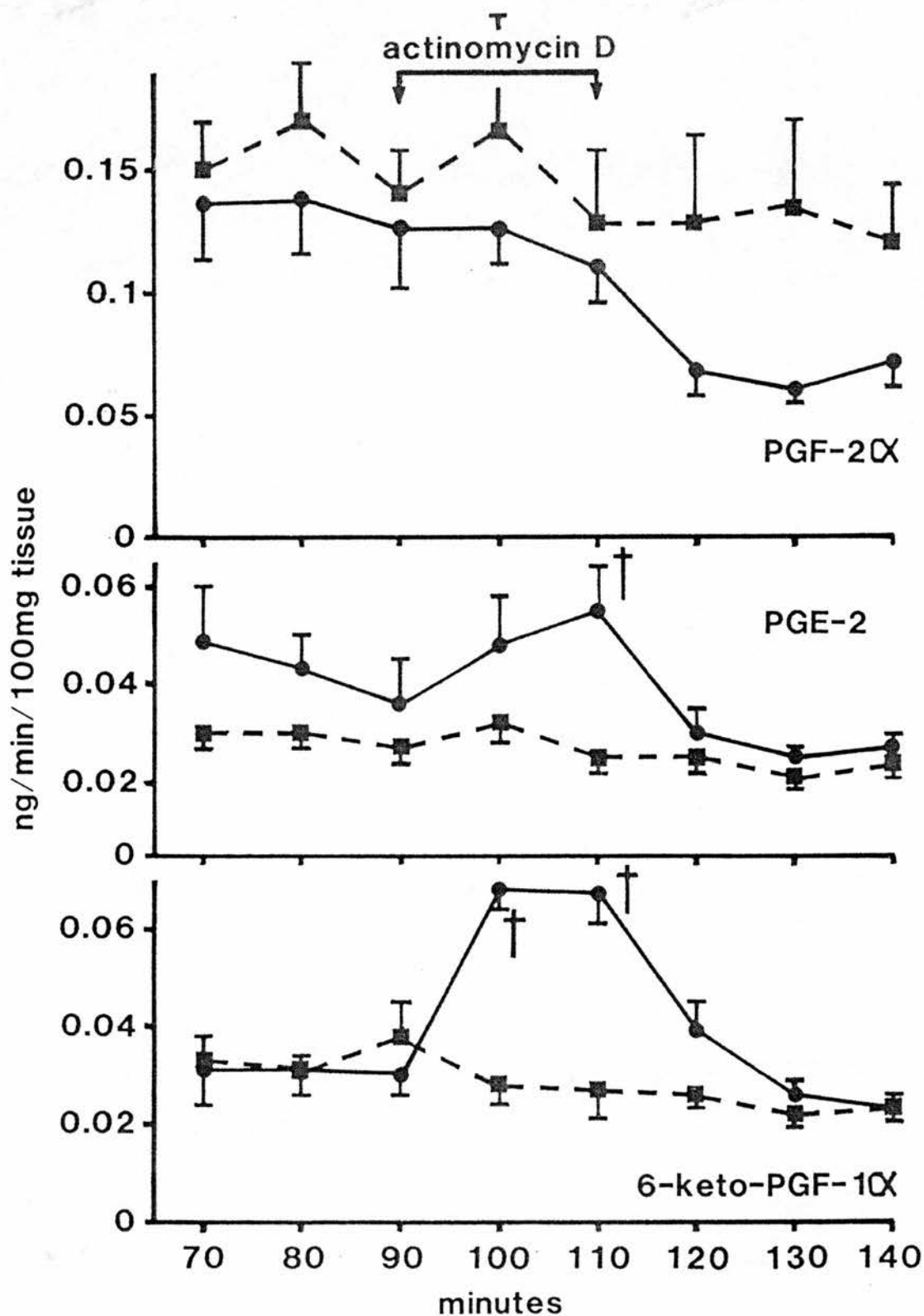


Figure 3.5.2.b

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 15 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\blacksquare - - \blacksquare$ ) or presence (treatment (T);  $\bullet - - \bullet$ ) of actinomycin D ( $50\mu\text{g/ml}$ ) between 90 min and 110 min.

† significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

exhibited no effect on  $\text{PGF}_{2\alpha}$  output from day 15 superfused uterine horns (Figure 3.5.2.d). The output of  $\text{PGE}_2$  was significantly ( $P < 0.05$ ) stimulated by cycloheximide during the treatment period, from both day 7 and day 15 superfused uterine horns and also during the 10 min sample period after treatment (sample 6) from day 15 superfused uterine horns (Figures 3.5.2.c and 3.5.2.d, respectively). Treatment with cycloheximide significantly ( $P < 0.05$ ) stimulated 6-keto- $\text{PGF}_{1\alpha}$  output from day 7 superfused uterine horns during the 20 min treatment period (Figure 3.5.2.c), and during the first 10 min of treatment from day 15 superfused uterine horns (Figure 3.5.2.d).

No significant change occurred in outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from day 7 (Figure 3.5.2.e) and day 15 (Figure 3.5.2.f) superfused uterine horns treated with puromycin. However, puromycin treatment significantly ( $P < 0.05$ ) stimulated 6-keto- $\text{PGF}_{1\alpha}$  output from day 7 uterine horns during the first 10 min (sample 4) of treatment, and stimulated 6-keto- $\text{PGF}_{1\alpha}$  output from day 15 superfused uterine horns throughout the 20 min treatment period (samples 4 and 5; Figures 3.5.2.e and 3.5.2.f, respectively).

The  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacities of endometrium and myometrium measured from uteri after 140 min of superfusion were not significantly altered by superfusing the uteri with actinomycin D ( $50\mu\text{g/ml}$ ; Figure 3.5.2.g), cycloheximide ( $50\mu\text{g/ml}$ ; Figure 3.5.2.h) and puromycin ( $50\mu\text{g/ml}$ ; Figure 3.5.2.i) between 90 and 110 min of the superfusion period, when compared to their respective untreated control values for the same PG synthesising capacity measured in the same tissue type removed on the same day of the oestrous cycle.



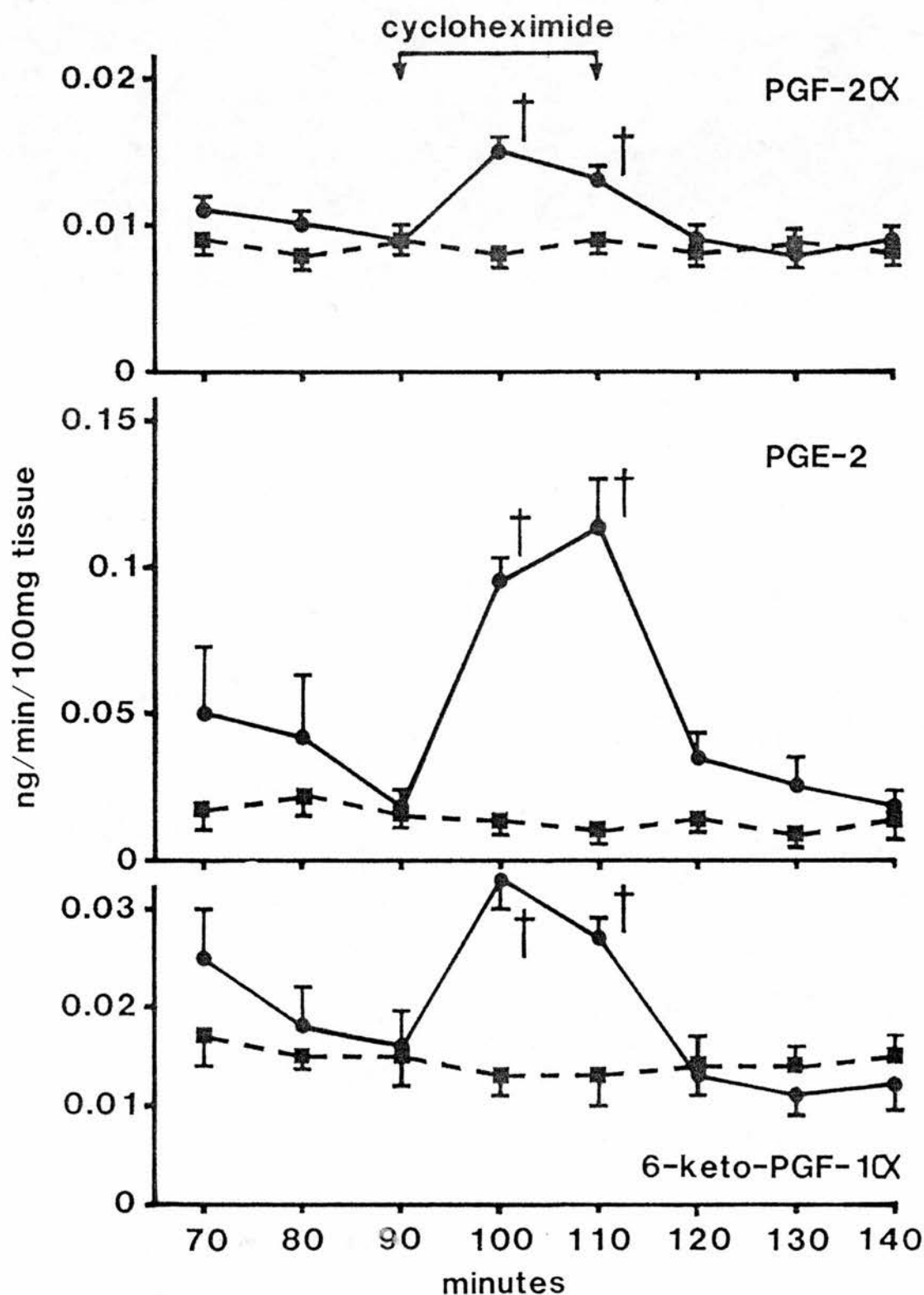


Figure 3.5.2.c

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of  $\text{PGF}_2\alpha$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\blacksquare - \blacksquare$ ) or presence (treatment (T);  $\bullet - \bullet$ ) of cycloheximide ( $50\mu\text{g/ml}$ ) between 90 min and 110 min.

† significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

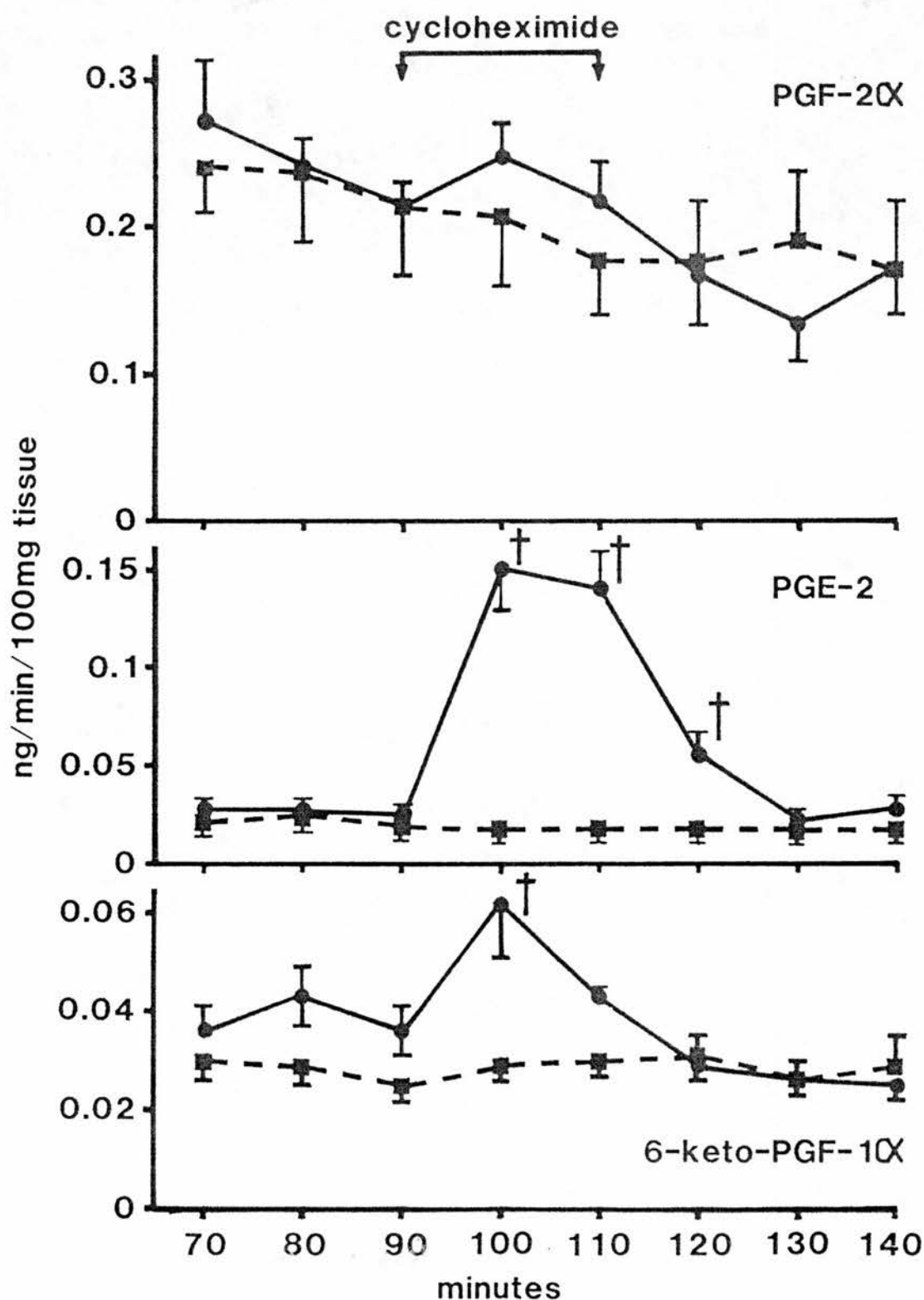


Figure 3.5.2.d

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 15 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\blacksquare - \blacksquare$ ) or presence (treatment (T);  $\bullet - \bullet$ ) of cycloheximide ( $50\mu\text{g/ml}$ ) between 90 min and 110 min.  
 $\dagger$  significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

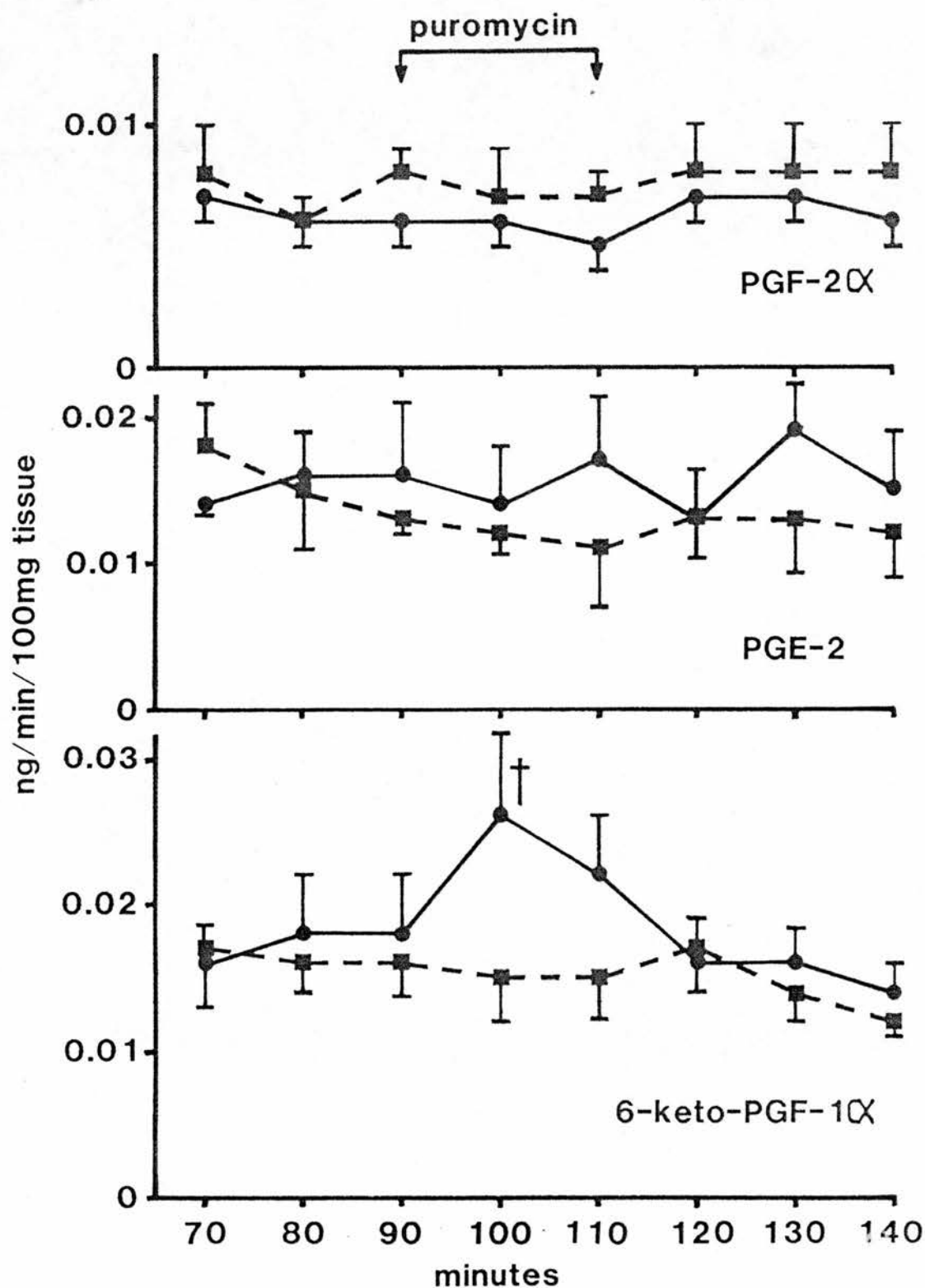


Figure 3.5.2.e

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\blacksquare - \blacksquare$ ) or presence (treatment (T);  $\bullet - \bullet$ ) of puromycin ( $50\mu\text{g/ml}$ ) between 90 min and 110 min.

† significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

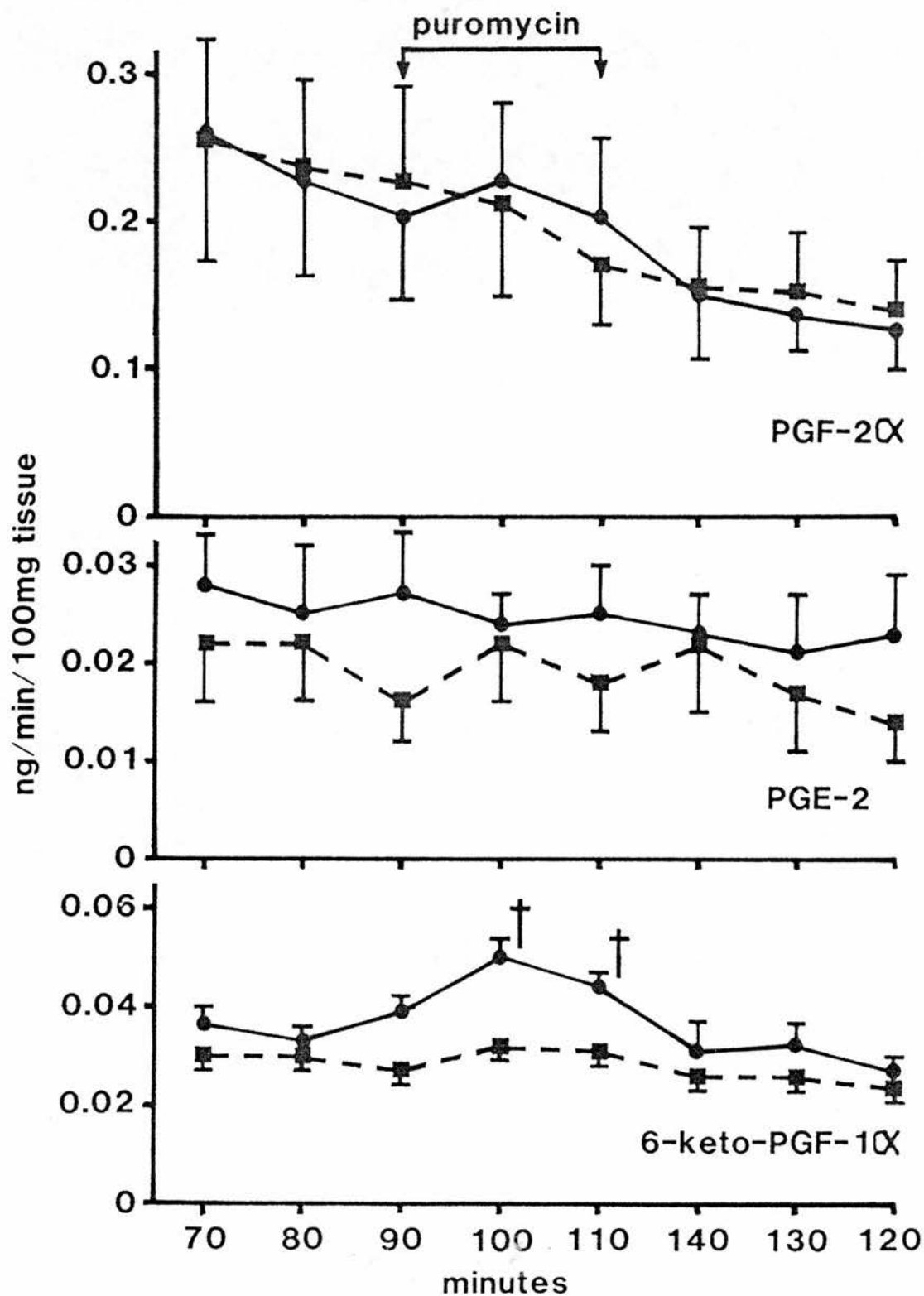


Figure 3.5.2.f

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 15 guinea-pig uterine horns superfused for 140 min, in the absence (control (C); broken line  $\blacksquare - \blacksquare$ ) or presence (treatment (T);  $\bullet - \bullet$ ) of puromycin ( $50\mu\text{g/ml}$ ) between 90 min and 110 min.

† significantly ( $P < 0.05$ ) higher than control values for the same PG prior to treatment.

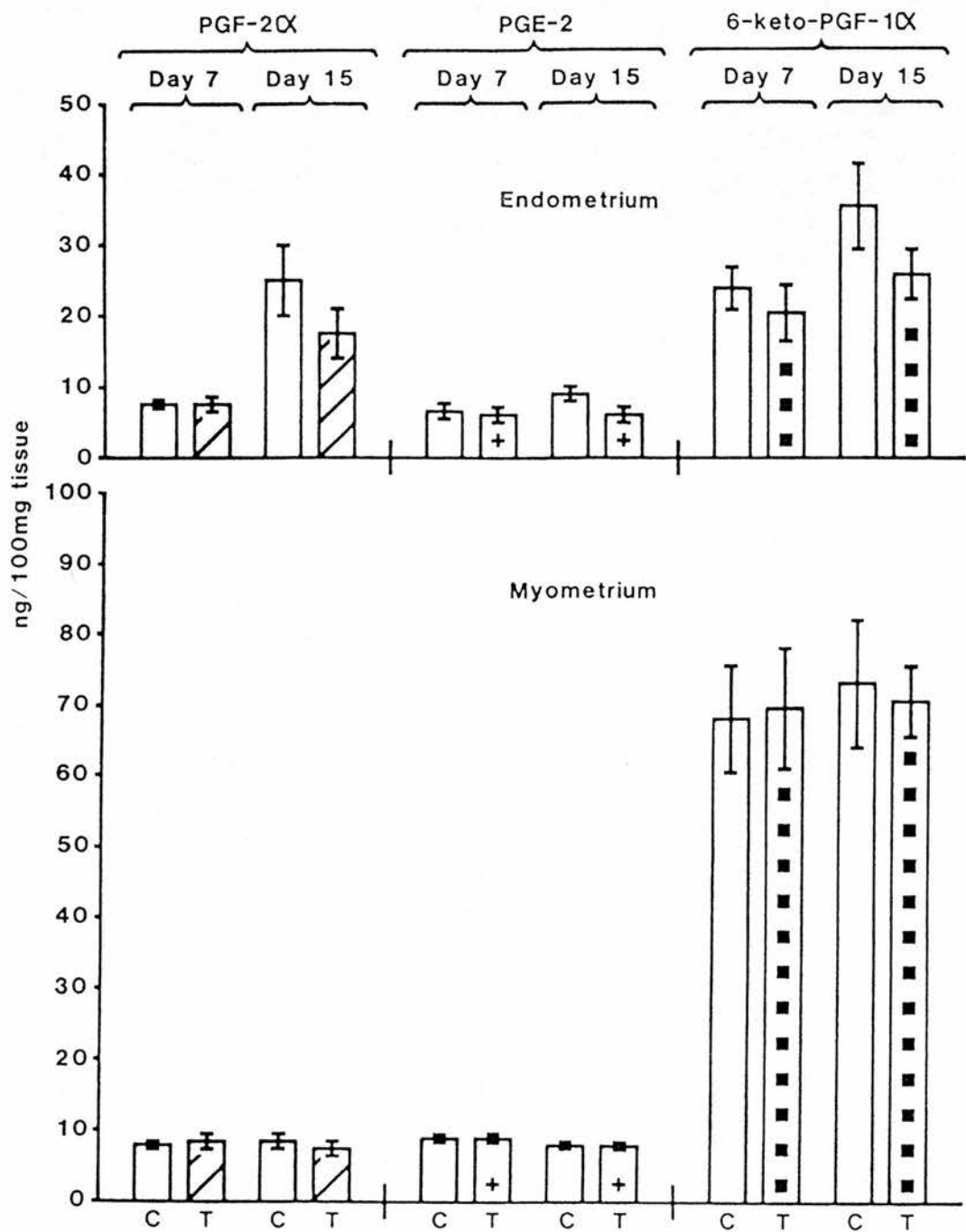


Figure 3.5.2.g

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesised by homogenates of endometrium and myometrium dissected from day 7 and day 15 guinea-pig uteri after superfusion for 140 min, and not treated (controls (C); open columns) or treated, (columns containing symbols) between 90 min and 110 min of superfusion with actinomycin D ( $50\mu\text{g/ml}$ ).

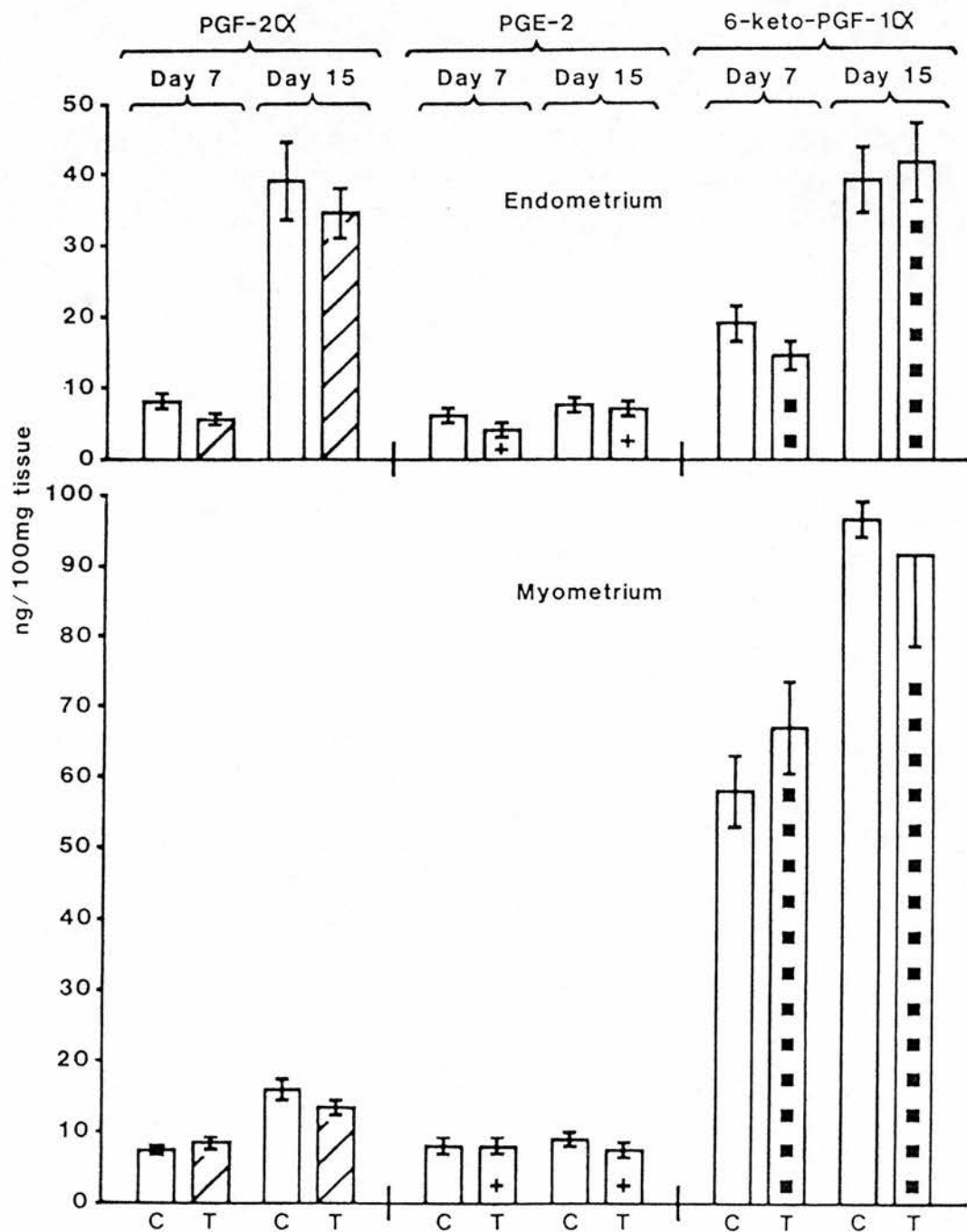


Figure 3.5.2.h

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts (ng/min/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  synthesised by homogenates of endometrium and myometrium dissected from day 7 and day 15 guinea-pig uteri after superfusion for 140 min, and not treated (controls (C); open columns) or treated (columns containing symbols) between 90 min and 110 min of superfusion with cycloheximide ( $50\mu\text{g/ml}$ ).

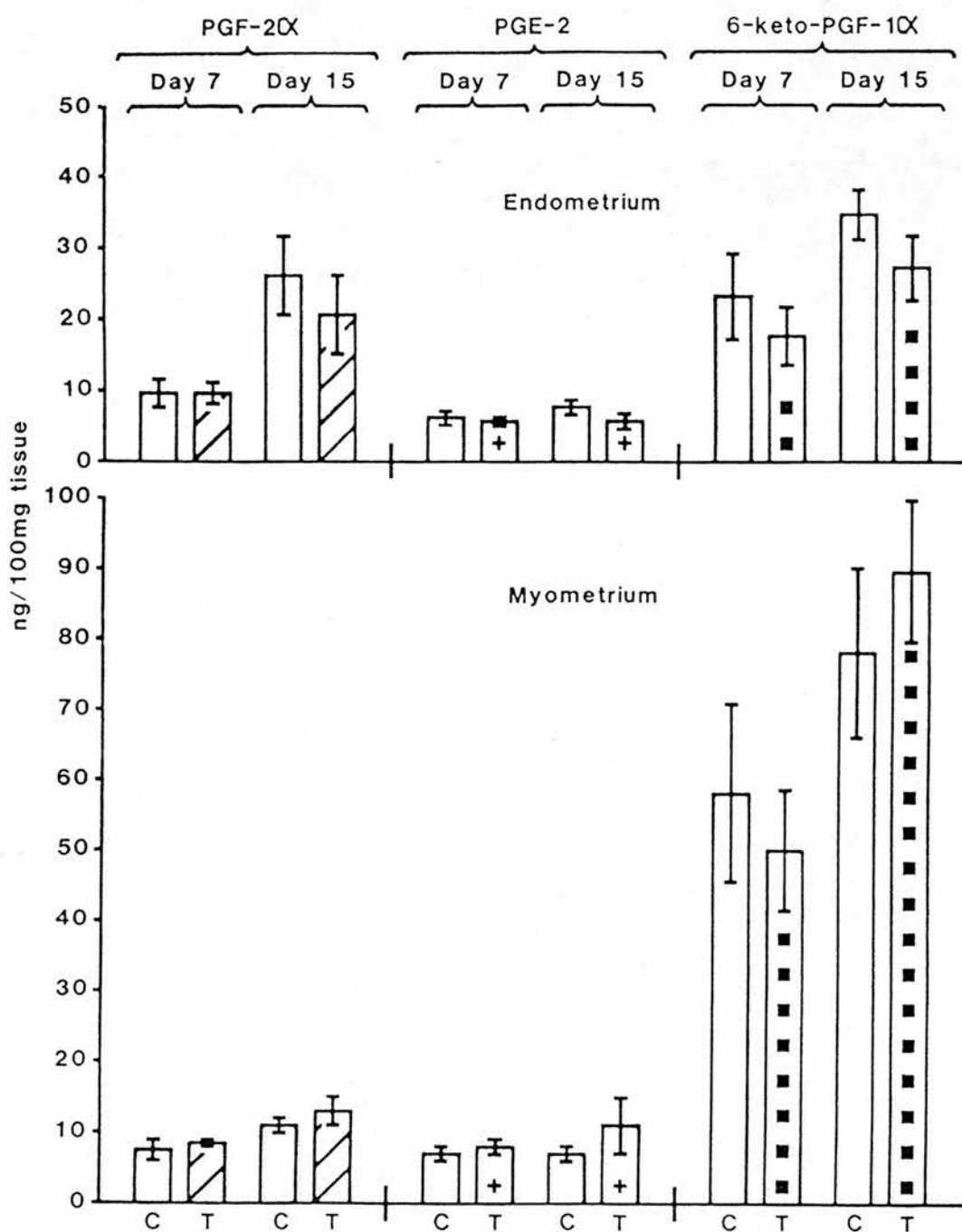


Figure 3.5.2.i

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts (ng/min/100mg tissue) of  $\text{PGF}_2\alpha$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesised by homogenates of endometrium and myometrium dissected from day 7 and day 15 guinea-pig uteri after superfusion for 140 min, and not treated (Controls (C); open columns) or treated (columns containing symbols) between 90 min and 110 min of superfusion with puromycin ( $50\mu\text{g/ml}$ ).

## CONCLUSIONS

Actinomycin D, puromycin and cycloheximide had no inhibitory action on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from either day 7 or day 15 uteri superfused in vitro for a short period of time. Furthermore, these protein or RNA synthesis inhibitors had no effect on the PG synthesising capacities of the myometrium and endometrium when measured approximately 1h after treatment. These results indicate that, in the guinea-pig uterus, there is no rapid inhibitory action of protein or RNA synthesis inhibitors on the synthesis of PGH synthase.

Paradoxically, actinomycin D treatment stimulated  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  output from the day 7 uterus and  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from the day 15 uterus. Cycloheximide treatment stimulated the outputs of all three PGs from both day 7 and day 15 uteri. The greatest stimulation was exhibited by cycloheximide treatment on  $\text{PGE}_2$  output, which showed a 5.3-fold increase from day 7, and 5.8-fold increase from day 15 superfused uteri. Puromycin treatment stimulated 6-keto- $\text{PGF}_{1\alpha}$  from both day 7 and day 15 uteri.

The mechanisms by which actinomycin D, cycloheximide and puromycin stimulate uterine PG synthesis need to be elucidated. However, it is clear that the protein and RNA synthesis inhibitors do not exert rapidly occurring inhibitory effects on PG output which has been reported in other tissues. Furthermore, the protein and RNA synthesis inhibitors do not exhibit a rapidly occurring, inhibitory action on endometrial or myometrial PG synthesizing capacity (a measure of tissue PGH synthase levels), an effect which in other tissues has been suggested to inhibit PG output.



### 3.5.3 THE EFFECT OF ACTINOMYCIN D TREATMENT IN VIVO ON PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG UTERUS

#### INTRODUCTION

A previous study by Poyser (1979) showed that actinomycin D, when injected into the lumen of the uterus, prevented the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity of the uterus that normally occurs towards the end of the oestrous cycle. This treatment also prevented luteal regression, as shown by the maintenance of high plasma progesterone concentrations, and increased the length of the oestrous cycle. In this study by Poyser (1979) it was proposed that  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus on day 15 had been inhibited by intra-uterine actinomycin D treatment on day 10. The present study has further investigated the effect of actinomycin D treatment in vivo on PG production by the guinea-pig uterus, and examined some possible regulatory mechanisms by which PG production may be controlled.

#### METHODS

Virgin, female guinea-pigs were used which had exhibited at least two oestrous cycles of normal length (section 2.2.1). After receiving the appropriate treatment, as detailed below none of the animals used exhibited any adverse effects from any of the treatments administered. All the animals were killed on day 15 of the oestrous cycle; a blood sample was collected from each of the animals and the uteri were removed (section 2.2.1). Plasma was separated from each blood sample by centrifugation, and was stored at  $-20^{\circ}\text{C}$  before the amount of progesterone present was measured by radioimmunoassay (section 2.3.5). The uteri were used in one of the following experiments.

## Experiment 1

Each uterus from five day 15 guinea-pigs was divided into its two uterine horns. One uterine horn was quickly dissected to separate the endometrium from the myometrium. Each tissue was weighed, homogenised and incubated for 60 min in the conditions as specified in section 2.2.4.i. These homogenates measured the "0 min" PG synthesizing capacities of their respective tissues. The other uterine horn was weighed, "opened" and prepared for superfusion (section 2.2.3). This horn was superfused with Krebs' solution (5ml/min) for 70 min, according to the method and conditions explained in section 2.2.3, and superfusate samples were taken every 10 min throughout the period of superfusion (i.e. 7 samples). Immediately after the period of superfusion, the endometrial layer was dissected from the myometrial layer and each tissue type was weighed, homogenised and incubated for 60 min as detailed for the "0 min" homogenate above. These homogenates measured the PG synthesizing capacities of their respective tissues at "70 min".

PGs were extracted from superfusate and incubate samples as explained in section 2.2.2, and the extracts were stored at  $-20^{\circ}\text{C}$  before the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present were measured by radioimmunoassay (section 2.3).

## Experiment 2

Ten day 10 guinea-pigs were anaesthetized by the intra-peritoneal injection of fentanyl citrate (0.47mg/kg) and fluanisone (15mg/kg; 'Hypnorm'), plus midazolam (3mg/kg; 'Hypnovel'). An incision was made in the abdomen of each guinea-pig, and then a thread was passed behind the junction of the two uterine horns. By pulling on this thread the junction of the two horns was easily exposed. Sterile saline solution (0.5ml; 9g NaCl/l) was injected into the

lumen of each uterine horn of five of the animals. The remaining five animals received an injection into each uterine horn of sterile saline (0.5ml) containing 20 $\mu$ g actinomycin D. The thread was then pulled free. The body wall and the skin of each guinea-pig were then stitched. Each animal then received a subcutaneous injection of naloxone (0.4mg; 'Narcan') which hastened the rate of recovery from the anaesthetic.

The uterus from each guinea-pig was removed on day 15, and the uterine horns were divided and treated according to a similar procedure as in Experiment 1. The two tissues from one uterine horn were homogenised and incubated for 60 min in order to measure the "0 min" PG synthesizing capacity. The other horn was superfused for 70 min as in Experiment 1. However, only two superfusate samples were taken from the superfused horn. The first sample was collected during the initial 10 min of superfusion (0-10 min), and the second sample was collected during the last 10 min of the 70 min superfusion period (60-70 min). After superfusion, the uterus was removed and the endometrium and myometrium were dissected apart, and were then weighed, homogenised and incubated for 60 min as explained in section 2.2.4.i. These homogenates measured the PG synthesizing capacities of the two tissues after "70 min". The PGs in the incubate and superfusate samples were extracted and stored at -20°C before the amounts of PGs present in the samples were measured by radioimmunoassay as in Experiment 1.

This procedure was repeated on a further ten guinea-pigs, with additionally each animal receiving a subcutaneous injection of 10 $\mu$ g oestradiol benzoate in 0.5ml arachis oil daily on day 11-14 of the oestrous cycle.

### Experiment 3

Ten day 10 guinea-pigs were anaesthetized and either saline (0.5ml) or saline (0.5ml) containing 20 $\mu$ g actinomycin D was injected into each uterine horn (five animals receiving each treatment), as explained in Experiment 2. On day 15, the uterus from each animal was removed and separated into its two horns, which were weighed, "opened" and prepared for superfusion as in Experiment 1. Both horns were superfused separately with Krebs' solution as described in section 2.2.3. After an initial settling period of 60 min, superfusate samples were collected every 10 min for the next 90 min from each horn (i.e. 9 samples/horn). One of these uterine horns from each animal was superfused with Krebs' solution containing A23187 (1 $\mu$ g/ml) during the collection of samples 4, 5 and 6 (test horn). The other horn remained untreated throughout the superfusion period (control horn). Immediately after the end of the superfusion, each horn was separated by dissection into its endometrium and myometrium, which were then weighed, homogenised and incubated for 60 min as in Experiment 1. These homogenates measured the PG synthesizing capacities for both tissues after 150 min of superfusion, with or without A23187 treatment. PGs in these incubate and superfusate samples were extracted and stored at -20°C before the amounts of PGs present in the samples were measured by radioimmunoassay as in Experiment 1.

This procedure was repeated on a further ten guinea-pigs, with additionally each animal receiving a subcutaneous injection of 10 $\mu$ g oestradiol benzoate in 0.5ml arachis oil daily on days 11-14 of the oestrous cycle.

### Experiment 4

Ten day 10 guinea-pigs were anaesthetized and each uterine horn

was injected with either saline (0.5ml) or saline (0.5ml) containing 20 $\mu$ g actinomycin D, as in Experiment 2, with five animals receiving each treatment. The uterus from each guinea-pig was removed on day 15 in preparation for tissue culture (section 2.2.1). The endometrium was separated from the myometrium and small pieces of endometrium were placed in three Petri dishes according to the tissue culture method (section 2.2.5). Two of these dishes contained 12-20mg of endometrium. The third dish contained 30-60mg of endometrium and 10 $\mu$ Ci [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml) was added to the medium, according to the method to investigate protein synthesis as described in section 2.4. Two further dishes were prepared for culture, each containing small pieces of myometrium weighing 15-25mg in total. These five dishes were placed in a modified Kilner jar, which was gassed and incubated for 24h as detailed in section 2.2.5.

Immediately after the jar was placed in the incubator, 30-60mg of the remaining endometrium were placed in a Petri dish containing 10 $\mu$ Ci [ $^3$ H]-leucine (2.5 $\mu$ Ci/ml), according to the tissue culture method (section 2.2.5). This tissue was washed with the [ $^3$ H]-leucine-containing medium and incubated for 2 min at room temperature. The tissue was then weighed. The tissue and medium were subjected to the methods, detailed in section 2.4, to quantify [ $^3$ H]-leucine incorporation into cellular and secreted proteins in order to measure the non-specific binding of the [ $^3$ H]-leucine that occurred during these procedures.

After 24h, the Kilner jar was removed from the incubator. The endometrium in the dish containing [ $^3$ H]-leucine was weighed, and the amounts of [ $^3$ H]-leucine incorporated into cellular and secreted proteins by the endometrium were measured by the procedures detailed in section 2.4.

The endometrium and myometrium from the remaining four dishes were weighed; the samples of culture medium were removed and stored at  $-20^{\circ}\text{C}$  before the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present were measured by radioimmunoassay (section 2.3).

#### Statistical tests and calculations

Duncan's multiple range test was used to test for significant changes in the output of a PG during the period of superfusion. All other comparisons were made using Student's 't' test, (which was used in a modified form if the variances were unequal as shown by the variance ratio F test), or by using the paired 't' test as appropriate.

The amounts of  $[^3\text{H}]$ -leucine which were bound non-specifically during the methods used for investigating cellular and secreted protein synthesis were subtracted from the respective results measuring  $[^3\text{H}]$ -leucine incorporation into cellular and secreted proteins obtained after 24h of culture.

### RESULTS

#### Experiment 1

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig uterine horns superfused in vitro for 70 min were high for the first ten minute sample period (Figure 3.5.2.a). The outputs of all three PGs then significantly ( $P < 0.05$ ) decreased between 10 and 20 min to a level of output that was 50-80% lower than the output from the first sample period. There was no significant change in the output from the uterine horns from this second sample throughout the remainder of the 70 min superfusion period, for any PG. The synthesizing capacities of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , but not of 6-keto- $\text{PGF}_{1\alpha}$ , in homogenates of endometrium significantly ( $P < 0.05$ ) decreased between 0 min and 70

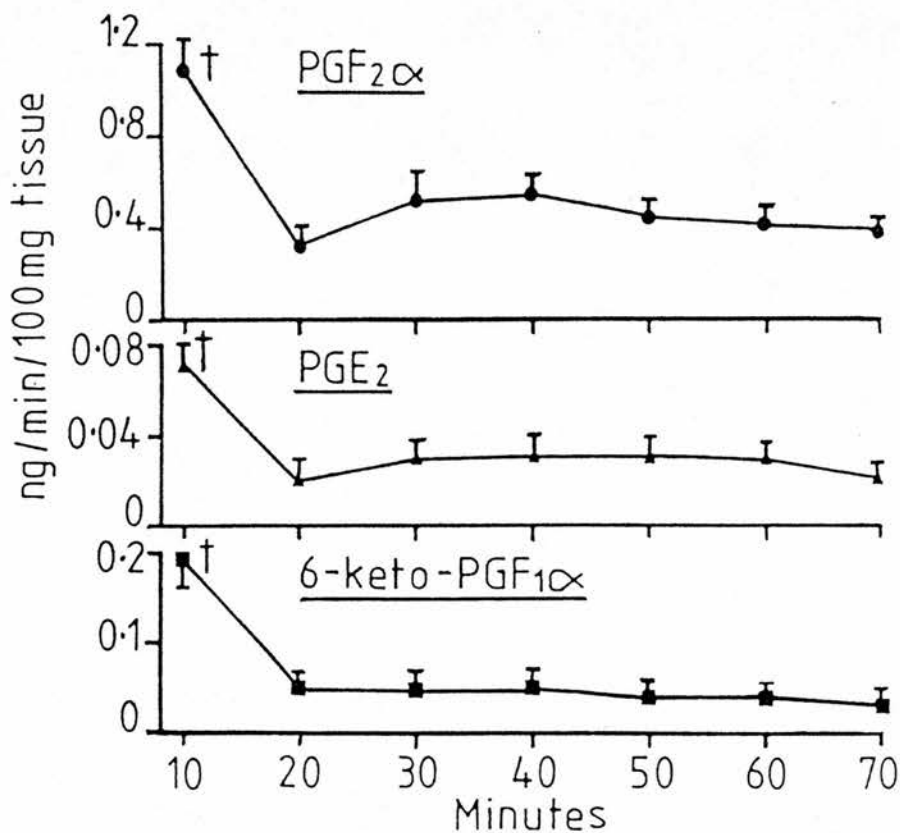


Figure 3.5.3.a

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α from day 15 guinea-pig uterine horns superfused in vitro.

† significantly ( $P < 0.05$ ) higher than the subsequent PG output value for the same PG.

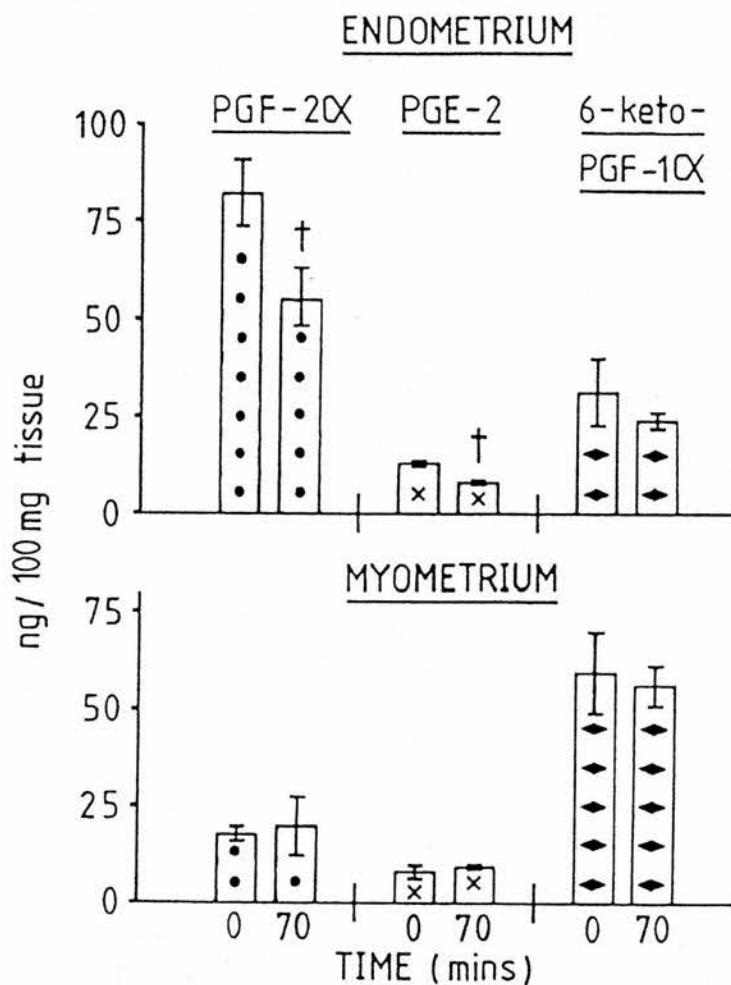


Figure 3.5.3.b

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts (ng/100mg tissue) of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesised by homogenates of endometrium and myometrium from day 15 guinea-pigs immediately after killing and removing the uterus from the animal (Time 0) or after 70 min superfusion.

† significantly ( $P < 0.05$ ) lower than corresponding value at 0 min for the same PG and tissue.



min (Figure 3.5.3.b). The synthesizing capacities of all three PGs in homogenates of myometrium did not alter between the two time periods. In this group of day 15 guinea-pigs, mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations were  $0.27 \pm 0.05$  ng/ml.

## Experiment 2

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from all uterine horns, not treated or treated with actinomycin D and not treated or treated with oestradiol benzoate between day 11 and day 14, were significantly ( $P < 0.05$ ) lower at 70 min than at 10 min (Figures 3.5.3.c and 3.5.3.d). The outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , but not of 6-keto- $\text{PGF}_{1\alpha}$ , were significantly ( $P < 0.05$ ) reduced from uterine horns treated with actinomycin D when compared to control horns after both 10 min and 70 min of superfusion (Figure 3.5.3.c). Similarly, the outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , but not of 6-keto- $\text{PGF}_{1\alpha}$ , were also significantly ( $P < 0.05$ ) lower at both times from actinomycin D-treated horns than from control horns from guinea-pigs that were also administered oestradiol benzoate on days 11-14 (Figures 3.5.3.d). The exception to this was that the output of  $\text{PGE}_2$  from actinomycin D-treated uterine horns was significantly ( $P < 0.05$ ) reduced after 70 min, but not after 10 min of superfusion (Figure 3.5.3.d).

The synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  in endometrium from guinea-pigs treated with intra-uterine saline (controls), but not from animals treated with intra-uterine actinomycin D, were significantly ( $P < 0.05$ ) decreased between 0 min and after 70 min of superfusion (Figure 3.5.3.e). The endometrial synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  of oestradiol-treated guinea-pigs were not significantly altered between 0 min and 70 min, irrespective of whether the uterine horns were treated with saline or actinomycin D (Figure 3.5.3.f). The synthesizing

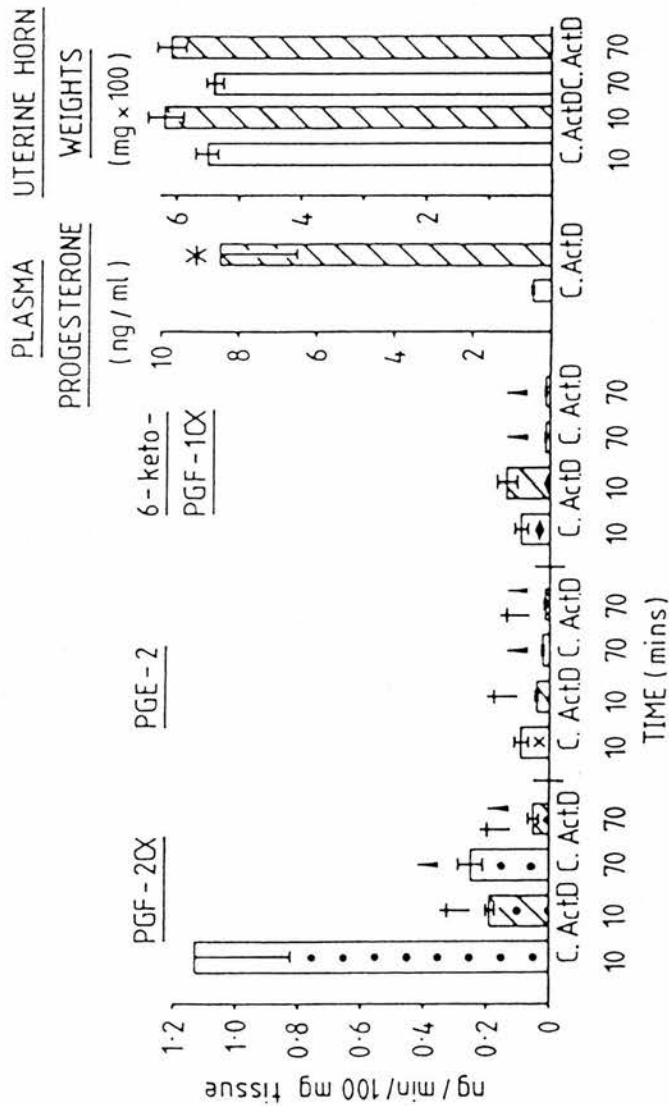


Figure 3.5.3.c

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of PGF<sub>2α</sub> (columns with dots), PGE<sub>2</sub> (columns with crosses) and 6-keto-PGF<sub>1α</sub> (columns with diamonds) released from superfused uterine horns (ng/min/100mg tissue) superfused for 10 min and 70 min, mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations (ng/ml) and mean ( $\pm$  s.e.m.;  $n = 5$ ) uterine horn weights (mg x 100) of day 15 guinea-pigs receiving on day-10 an intra-uterine injection into each horn of either NaCl solution (mg x 100) NaCl/1; controls (C) or NaCl solution containing actinomycin D (20μg actinomycin D/horn; hatched columns). † significantly ( $P < 0.05$ ) lower than corresponding control for the same PG at the same time. ▲ significantly ( $P < 0.05$ ) lower than corresponding output at 10 min for the same PG from the uterine horns receiving the same treatment. \* significantly ( $P < 0.05$ ) higher than control plasma progesterone concentration

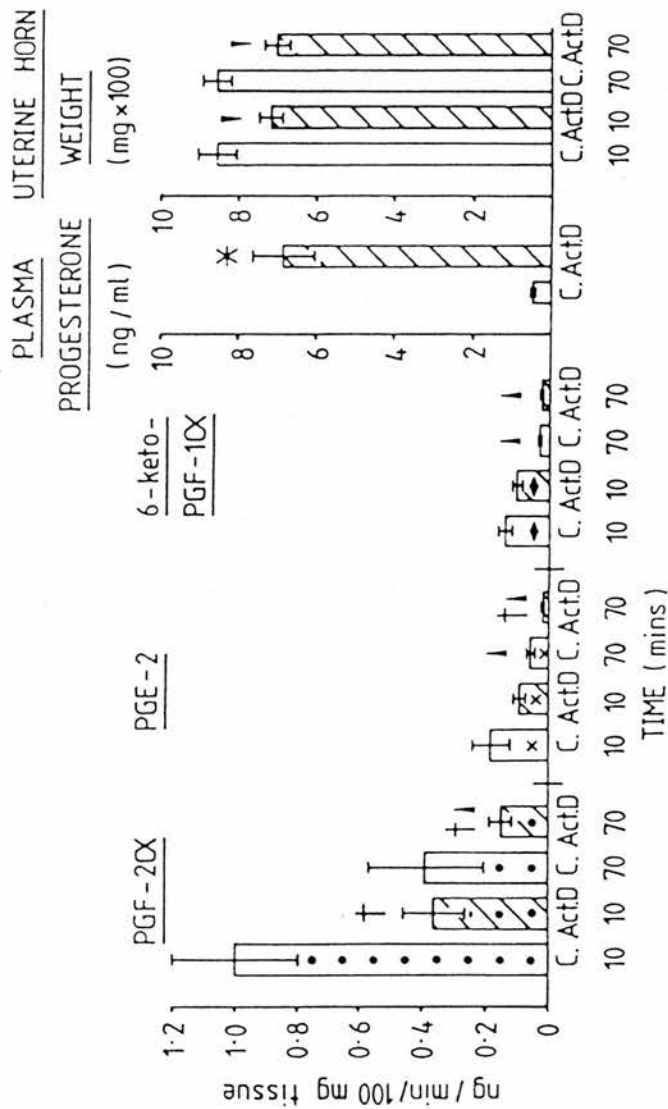


Figure 3.5.3.3.d

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of  $\text{PGF}_{2\alpha}$  (columns with dots),  $\text{PGE}_2$  (columns with crosses) and 6-keto- $\text{PGF}_{1\alpha}$  (columns with diamonds) released from superfused uterine horns ( $\text{ng/min/100mg}$  tissue) superfused for 10 min and 70 min, mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations ( $\text{ng/ml}$ ), and mean ( $\pm$  s.e.m.;  $n = 5$ ) uterine horn weights ( $\text{mg} \times 100$ ) of day 15 guinea-pigs receiving on day-10 an intra-uterine injection into each horn of either NaCl solution ( $0.5\text{ml}$ ;  $9\text{g}$  NaCl/l; controls (C)) or NaCl solution containing actinomycin D ( $20\mu\text{g}$  actinomycin D/horn; hatched columns) and treated with a subcutaneous injection of  $10\mu\text{g/ml}$  oestradiol benzoate daily on days 11-14.

† significantly ( $P < 0.05$ ) lower than corresponding control output for the same PG at the same time.

‡ significantly ( $P < 0.05$ ) lower than corresponding output at 10 min for the same PG from the uterine horns receiving the same treatment.

\* significantly ( $P < 0.05$ ) lower than corresponding control uterine horn weight.

\* significantly ( $P < 0.05$ ) higher than control plasma progesterone concentration.

capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  in the myometrium were not changed between 0 min and 70 min irrespective of whether the guinea-pigs received intra-uterine saline or intra-uterine actinomycin D on day 10, and were treated or not treated with oestradiol benzoate on days 11-14 (Figures 3.5.3.e and 3.5.3.f).

The amounts of  $\text{PGF}_{2\alpha}$ , but not of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , synthesised by homogenates of endometrium were significantly ( $P < 0.05$ ) reduced in animals receiving intra-uterine actinomycin D treatment, when compared to saline-treated controls (Figure 3.5.3.e). However, in oestradiol-treated guinea-pigs, the endometrial synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were all significantly ( $P < 0.05$ ) reduced by actinomycin D treatment when compared to saline-treated control animals (Figure 3.5.3.f). Intra-uterine actinomycin D treatment did not change PG synthesizing capacities in homogenates of myometrium from guinea-pigs not treated or treated with oestradiol benzoate between days 11-14 (Figures 3.5.3.e and 3.5.3.f).

In guinea-pigs that received intra-uterine actinomycin D, with or without oestradiol benzoate treatment, plasma progesterone concentrations on day 15 were significantly ( $P < 0.05$ ) higher compared to the low plasma progesterone concentrations in intra-uterine saline treated (control) animals (Figures 3.5.3.c and 3.5.3.d). Intra-uterine actinomycin D administration significantly ( $P < 0.05$ ) inhibited the increase in uterine horn weights induced by treatment with oestradiol benzoate (Figure 3.5.3.d).

### Experiment 3

The basal output of  $\text{PGF}_{2\alpha}$  from day 15 guinea-pig uterine horns superfused in vitro was significantly ( $P < 0.05$ ) inhibited by the intra-uterine administration of actinomycin D on day 10, with or

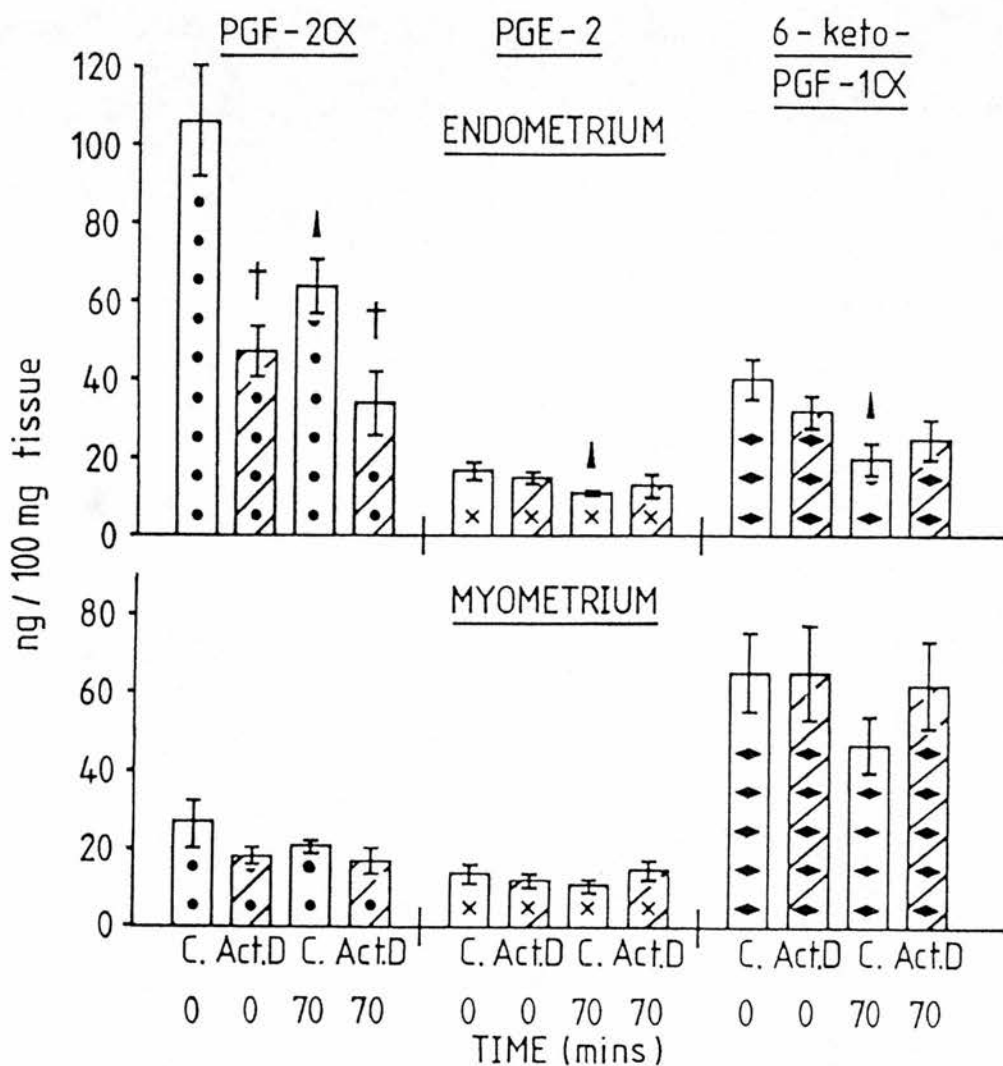


Figure 3.5.3.e

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of  $\text{PGF}_2\alpha$  (columns with dots),  $\text{PGE}_2$  (columns with crosses) and 6-keto- $\text{PGF}_{1\alpha}$  (columns with diamonds) synthesised (ng/100mg tissue) by homogenates of endometrium and myometrium from day 15 guinea-pigs receiving on day-10 an intra-uterine injection into each horn of either saline (control (C)) or saline containing  $20\mu\text{g}$  actinomycin D (hatched columns) and either not superfused (0 min) or superfused for 70 min.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG at the same time and from the same tissue.

▲ significantly ( $P < 0.05$ ) lower than corresponding value at 0 min for the same PG from the same tissue receiving the same treatment.

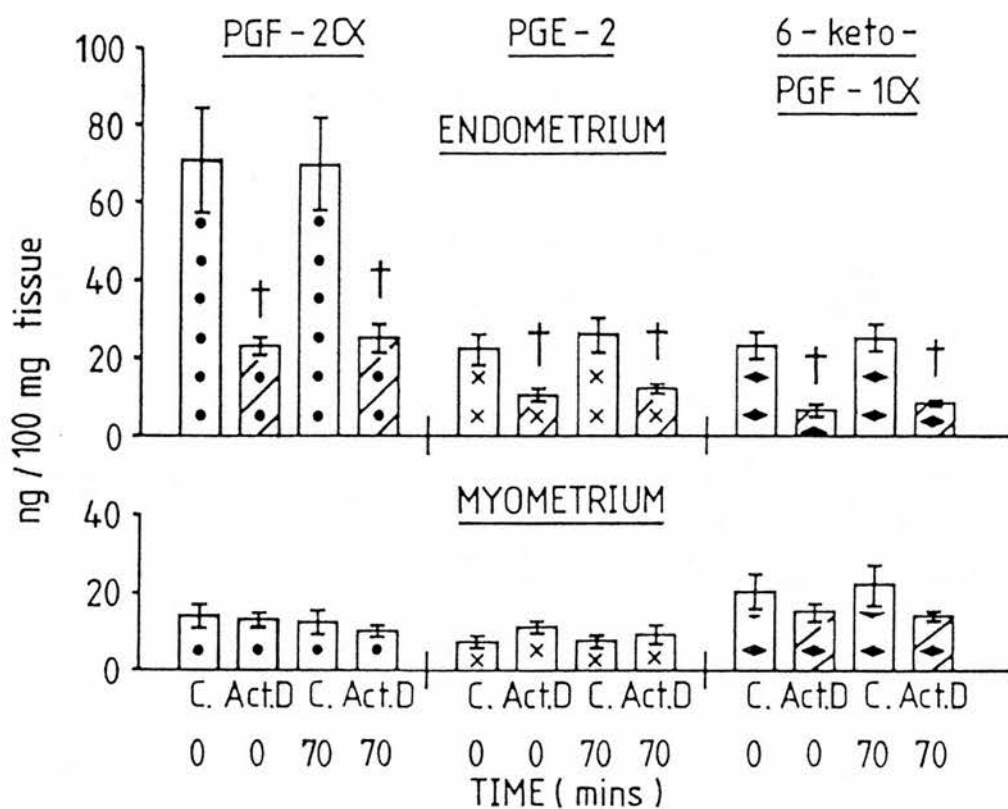


Figure 3.5.3.f

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of  $\text{PGF}_{2\alpha}$  (columns with dots),  $\text{PGE}_2$  (columns with crosses) and 6-keto- $\text{PGF}_{1\alpha}$  (columns with diamonds) synthesised (ng/100mg tissue) by homogenates of endometrium and myometrium from day 15 guinea-pigs receiving on day-10 an intra-uterine injection into each horn of either saline (control (C)) or saline containing  $20\mu\text{g}$  actinomycin D (hatched columns) and either not superfused (0 min) or superfused for 70 min, and treated with a subcutaneous injection of  $10\mu\text{g}$  oestradiol benzoate daily on days 11-14.

† significantly ( $P < 0.05$ ) lower than corresponding control value for the same PG at the same time and from the same tissue.

without oestradiol treatment on days 11-14 (Figures 3.5.3.g and 3.5.3.h). The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were also significantly ( $P < 0.05$ ) inhibited by intra-uterine treatment with actinomycin D on day 10, irrespective of whether the guinea-pigs were not treated or treated with oestradiol benzoate (Figures 3.5.3.g and 3.5.3.h). The outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$ , and to a lesser extent  $\text{PGE}_2$ , were significantly ( $P < 0.05$ ) stimulated by A23187 treatment and this stimulation was independent of the treatment in vivo (Figures 3.5.3.g and 3.5.3.h). A23187 treatment caused an approximately 3-fold stimulation of  $\text{PGF}_{2\alpha}$  output above basal outputs from the uterus of guinea-pigs treated in vivo with saline or actinomycin D and not receiving or receiving oestradiol benzoate between days 11-14 (Figures 3.5.3.g and 3.5.3.h).

The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesised by homogenates of endometrium and myometrium after 150 min superfusion were not changed by treatment with A23187 (Figures 3.5.3.i and 3.5.3.j). The synthesizing capacity of  $\text{PGF}_{2\alpha}$  but not of  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$ , was significantly ( $P < 0.05$ ) reduced in both the endometrium and myometrium of actinomycin D-treated uterine horns from guinea-pigs receiving oestradiol benzoate (Figure 3.5.3.i). The synthesizing capacities of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  in endometrium from oestradiol-treated guinea-pigs were significantly ( $P < 0.05$ ) reduced by actinomycin D treatment (Figure 3.5.3.j). Treatment with intra-uterine actinomycin D had no effect on PG synthesizing capacities of the myometrium from guinea-pigs receiving oestradiol benzoate on days 11-14 (Figure 3.5.3.j).

Mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations in actinomycin D-treated and saline-treated guinea-pigs, not receiving oestradiol benzoate, were  $4.13 \pm 0.52$  and  $0.21 \pm 0.04\text{ng/ml}$ ,

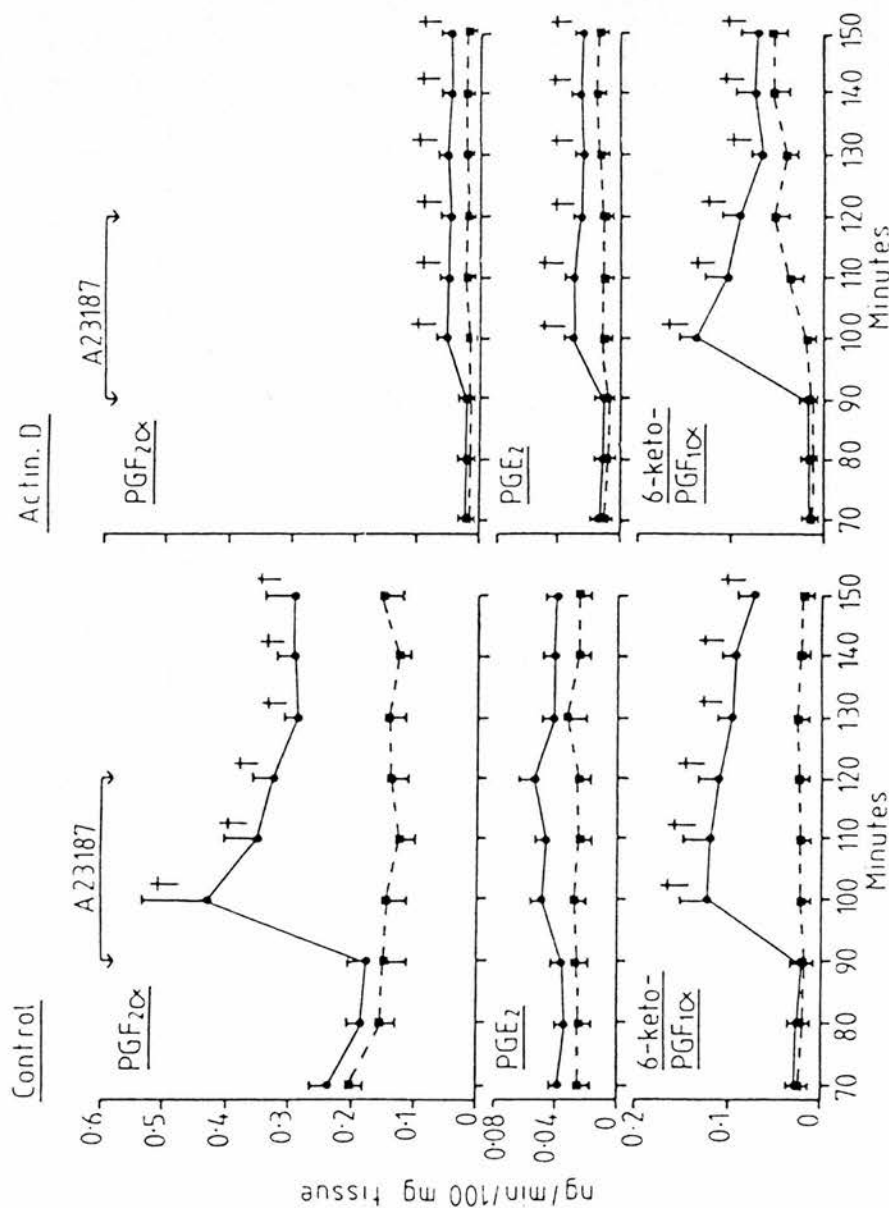


Figure 3.5.3.3.g  
Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs of prostaglandins (ng/min/100mg tissue) from the uterine horns superfused in vitro of day 15 guinea-pigs receiving on day 10 an intra-uterine injection into both horns of either saline (controls) or saline containing 20 $\mu$ g actinomycin D (Actin. D) and not treated (broken line) or treated (solid line) with A23187 (1 $\mu$ g/ml) in the superfusing medium as indicated.  
\* significantly ( $P < 0.05$ ) higher than corresponding values for the same PG before treatment with A23187.



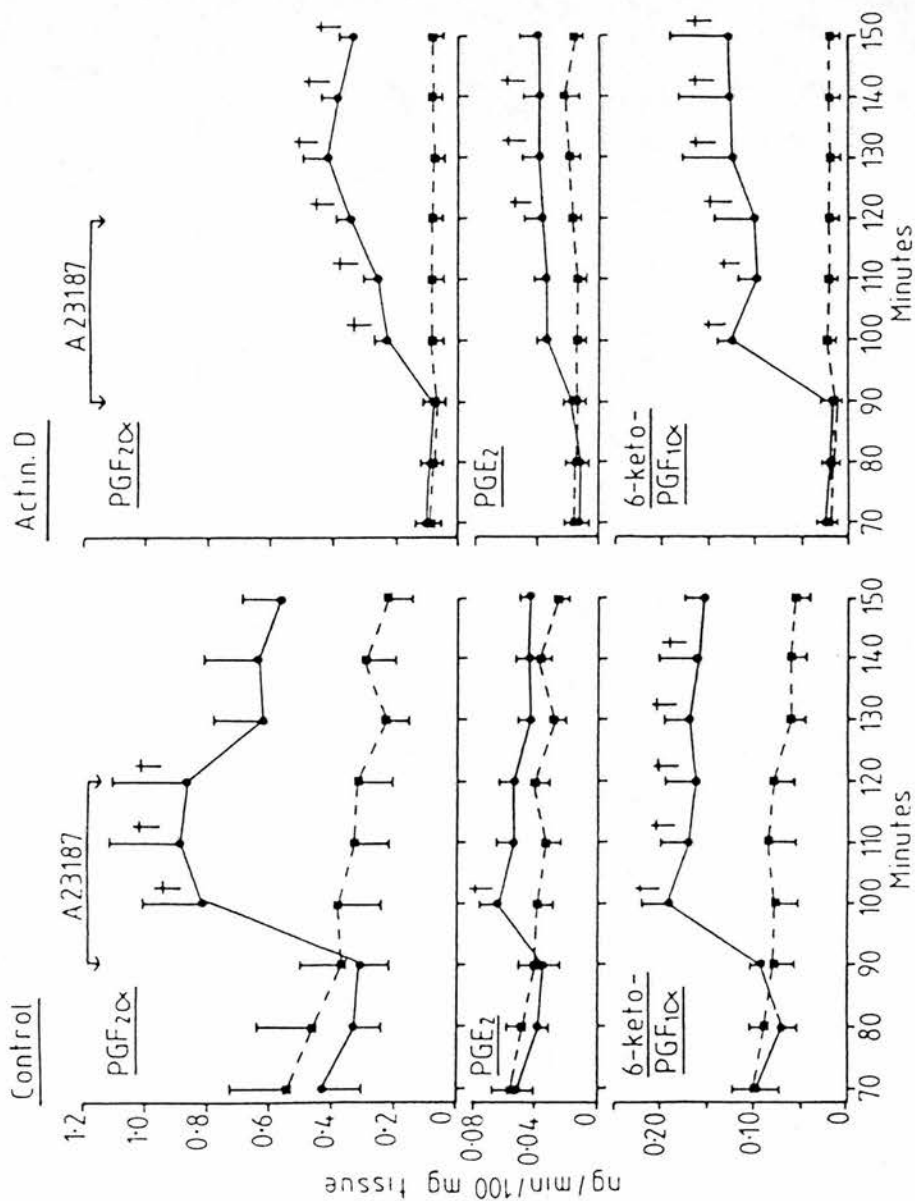


Figure 3.5.3.3.h  
Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs of prostaglandins (ng/min/100mg tissue) from the uterine horns superfused in vitro of day 15 guinea-pigs receiving on day 10 an intra-uterine injection into both horns of either saline (controls) or saline containing 20 $\mu$ g actinomycin D (Actin. D) and treated with a sub-cutaneous injection of 10 $\mu$ g oestradiol benzoate daily on days 11-14.  
† significantly ( $P < 0.05$ ) higher than corresponding values for the same PG before treatment with A23187.

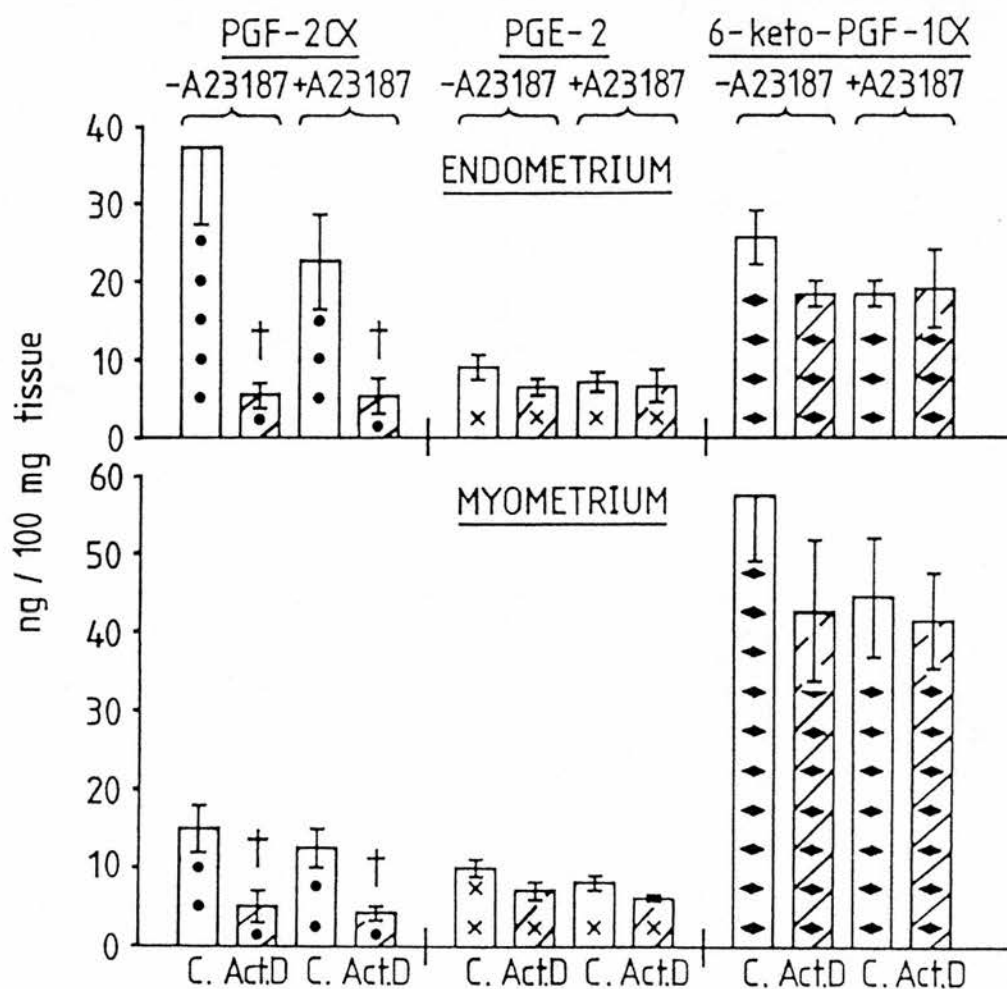


Figure 3.5.3.i

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of  $\text{PGF}_{2\alpha}$  (columns with dots),  $\text{PGE}_2$  (columns with crosses) and  $6\text{-keto-PGF}_{1\alpha}$  (columns with diamonds) synthesized (ng/100mg tissue) by homogenates of endometrium and myometrium from uterine horns superfused for 150 min from day 15 guinea-pigs receiving on day-10 an intra-uterine injection of either saline (control; C) or saline containing  $20\mu\text{g}$  actinomycin D (hatched columns; Act.D) and during 150 min superfusion of the uterine horns in vitro not treated (-) or treated (+) with A23187.

† significantly ( $P < 0.05$ ) lower than corresponding control values for the same PG from the same tissue receiving similar treatment in vivo and in vitro.

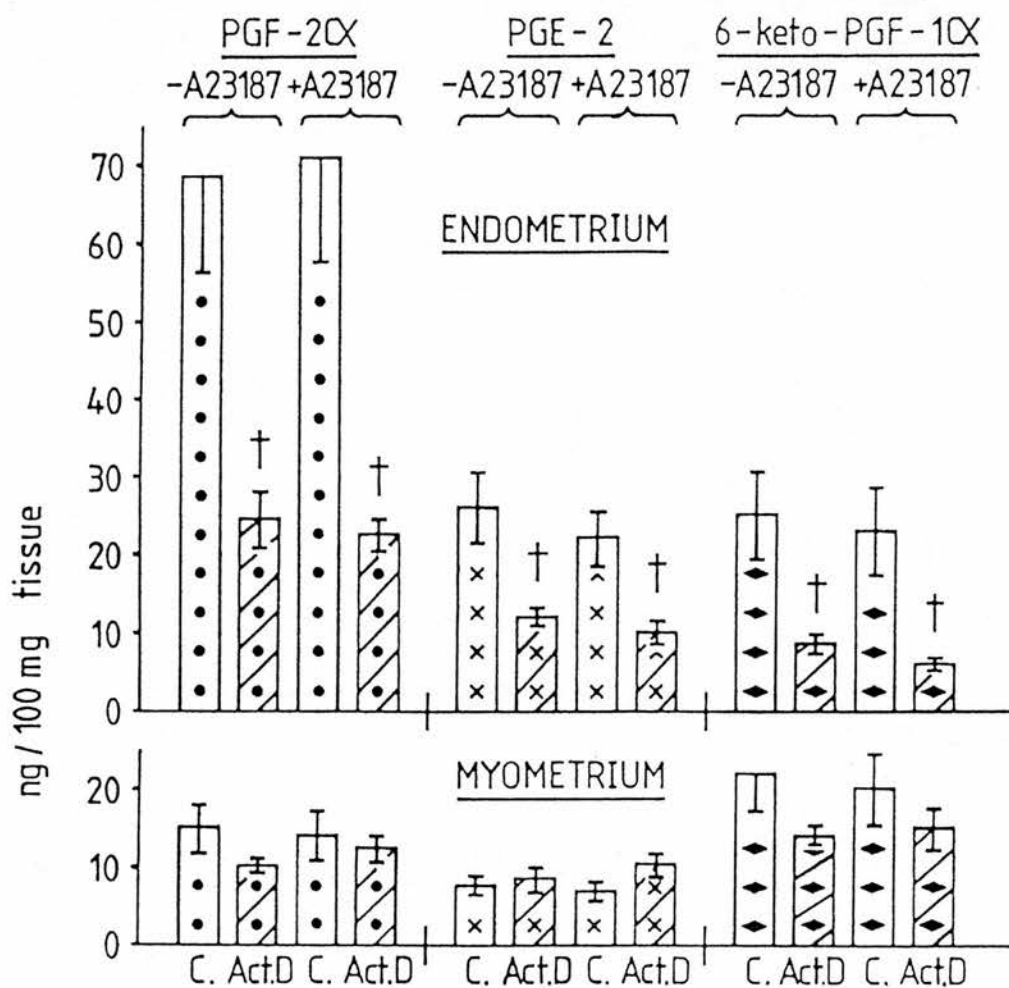


Figure 3.5.3.j

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of  $\text{PGF}_2\alpha$  (columns with dots),  $\text{PGE}_2$  (columns with crosses) and 6-keto- $\text{PGF}_{1\alpha}$  (columns with diamonds) synthesised (ng/100mg tissue) by homogenates of endometrium and myometrium from uterine horns superfused for 150 min from day 15 guinea-pigs receiving on day-10 an intra-uterine injection of either saline (control; C) or saline containing  $20\mu\text{g}$  actinomycin D (hatched columns; Act. D) and treated with a subcutaneous injection of  $10\mu\text{g}$  oestradiol benzoate daily on days 11-14 and during 150 min superfusion of the uterine horns in vitro not treated (-) or treated (+) with A23187.

† significantly ( $P < 0.05$ ) lower than corresponding control values for the same PG from the same tissue receiving similar treatment in vivo and in vitro.

respectively. In guinea-pigs receiving oestradiol benzoate on days 11-14 and treated with intra-uterine actinomycin D or saline, the mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations were  $3.31 \pm 0.80$  and  $0.11 \pm 0.02$  ng/ml, respectively. Actinomycin D treatment significantly ( $P < 0.05$ ) inhibited the increase in uterine horn weights induced by oestradiol benzoate treatment. The mean ( $\pm$  s.e.m.;  $n = 10$ ) weights of the oestradiol-treated and intra-uterine saline-treated, and oestradiol-treated and intra-uterine actinomycin D-treated uterine horns were  $910 \pm 43$  and  $596 \pm 24$  mg, respectively.

#### Experiment 4

The amounts of [ $^3$ H]-leucine incorporated into cellular and secreted proteins by day 15 endometrium obtained from uteri which had been treated with intra-uterine saline on day 10 (i.e. controls) were not significantly different from the values obtained for untreated day 15 endometrium presented in section 3.4 (Figure 3.4.a). The intra-uterine injection of actinomycin D on day 10 significantly ( $P < 0.05$ ) inhibited the amounts of [ $^3$ H]-leucine incorporated into cellular and secreted proteins by endometrium removed on day 15 and cultured for 24h (Figure 3.5.3.k).

The output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium removed from uteri which had received intra-uterine actinomycin D treatment on day 10 and cultured for 24h was significantly ( $P < 0.05$ ) reduced when compared to the control  $\text{PGF}_{2\alpha}$  output from endometrium removed from intra-uterine saline-treated uteri and cultured for 24h (Figure 3.5.3.l). The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 cultured endometrium from uteri receiving intra-uterine actinomycin D treatment in vivo on day 10 were not significantly altered compared to their corresponding control endometrial PG output values for uteri receiving intra-uterine saline treatment (Figure 3.5.3.l). The outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$

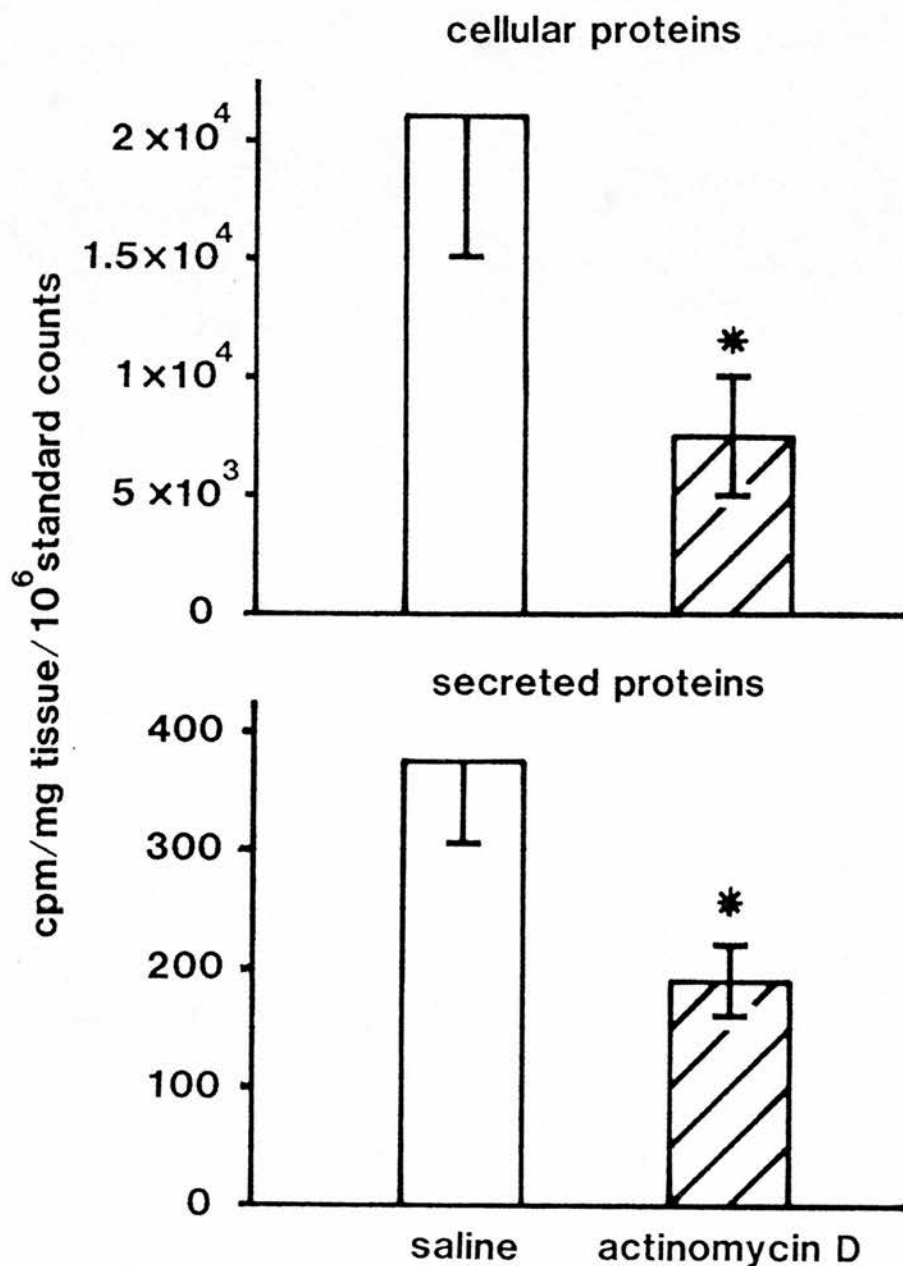


Figure 3.5.3.k

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of [<sup>3</sup>H]-leucine incorporated into cellular and secreted proteins by day 15 endometrium cultured for 24h after receiving either intra-uterine saline (controls; open columns) or intra-uterine saline containing actinomycin D (20 $\mu$ g actinomycin D/horn; hatched columns on day 10 of the oestrous cycle).

\* significantly ( $P < 0.05$ ) lower incorporation of [<sup>3</sup>H]-leucine into the same category of protein when compared to the endometrial proteins from intra-uterine saline injected (control) guinea-pigs

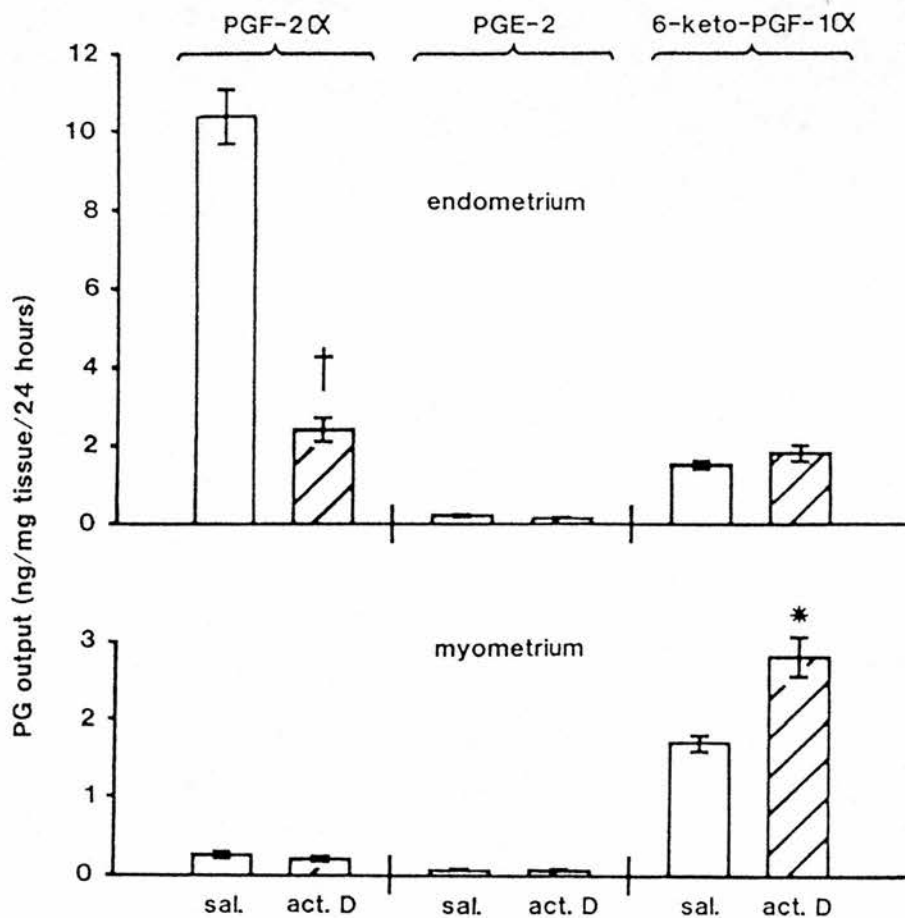


Figure 3.5.3.1

Mean ( $\pm$  s.e.m.;  $n = 5$ ) outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  from day 15 guinea-pig endometrium and myometrium cultured for 24h, receiving either intra-uterine saline (controls; sal.; open columns) or intra-uterine saline containing actinomycin D (20 $\mu$ g actinomycin D/horn; act.D; hatched columns) on day 10 of the oestrous cycle.

† significantly ( $P < 0.05$ ) lower than corresponding control (animal receiving saline treatment) PG output from the same tissue type.

\* significantly ( $P < 0.05$ ) higher than corresponding control (animal receiving saline treatment) PG output from the same tissue type.

from myometrium from intra-uterine actinomycin-D treated uteri and cultured for 24h were not significantly changed compared to their respective control myometrial PG outputs from uteri receiving intra-uterine saline treatment (Figure 3.5.3.1). However, 6-keto-PGF<sub>1α</sub> output from myometrium removed from uteri receiving intra-uterine actinomycin D treatment and cultured for 24h was significantly ( $P < 0.05$ ) stimulated 1.7-fold when compared to the corresponding control 6-keto-PGF<sub>1α</sub> output from cultured myometrium from uteri treated with intra-uterine saline (Figure 3.5.3.1).

Mean ( $\pm$  s.e.m.;  $n = 5$ ) plasma progesterone concentrations in actinomycin D-treated and saline-treated guinea-pigs in this experiment were  $3.99 \pm 0.49$  and  $0.59 \pm 0.10$  ng/ml, respectively.

## CONCLUSIONS

The syntheses of cellular and secreted proteins by cultured day 15 guinea-pig endometrium were inhibited by the intra-uterine administration of actinomycin D on day 10. The output of PGF<sub>2α</sub> from the superfused day 15 guinea-pig uterus was decreased by 80-85% by the intra-uterine administration of actinomycin D. Plasma progesterone concentrations were high in day 15 guinea-pigs receiving intra-uterine actinomycin D on day 10 when compared to control day 15 guinea-pigs receiving intra-uterine saline on day 10. The high plasma progesterone levels indicated that luteal regression had been prevented due to the inhibition of uterine PGF<sub>2α</sub> synthesis by actinomycin D treatment. Administration of oestradiol benzoate on days 11-14 did not overcome the inhibitory action of actinomycin D treatment (on day 10) on day 15 uterine PGF<sub>2α</sub> output. The inhibition of PGF<sub>2α</sub> output by actinomycin D treatment which was not overcome by oestradiol benzoate administration, was also reflected in the

maintenance of high plasma progesterone concentrations on day 15, indicating that the lifespan of the corpora lutea had been extended. The inhibitory action of actinomycin D treatment in vivo on day 15 guinea-pig uterine  $\text{PGF}_{2\alpha}$  output appeared selective to the endometrial layer of the uterus because, in actinomycin D-treated guinea-pigs,  $\text{PGF}_{2\alpha}$  output was inhibited from the endometrium but not from the myometrium when the tissues were cultured for 24h. The output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium cultured for 24h was 4.4-fold higher from saline-treated compared to actinomycin D-treated guinea-pigs.

The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the superfused guinea-pig uterus removed on day 15 after receiving intra-uterine saline (i.e. control) on day 10 were much lower than the output of  $\text{PGF}_{2\alpha}$  from the same superfused uterus. Treatment with intra-uterine actinomycin D reduced the output of  $\text{PGE}_2$ , but not of 6-keto- $\text{PGF}_{1\alpha}$ , from the day 15 superfused guinea-pig uterus, and this inhibitory action was not overcome by oestradiol benzoate treatment. The output of  $\text{PGE}_2$  from cultured endometrium taken from actinomycin D-treated uteri was inhibited by approximately 50% compared to  $\text{PGE}_2$  output from saline-treated uteri, although this effect was not significant due to the very low  $\text{PGE}_2$  outputs measured and hence the relatively larger standard errors observed. The output of  $\text{PGE}_2$  from myometrium in culture was not significantly altered by actinomycin D treatment in vivo. The output of 6-keto- $\text{PGF}_{1\alpha}$  from cultured endometrium from actinomycin D-treated horns was not changed significantly compared to saline-treated horns. Paradoxically, actinomycin D treatment in vivo stimulated 6-keto- $\text{PGF}_{1\alpha}$  output from myometrium in culture.

The endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity in guinea-pigs receiving intra-uterine saline (i.e. controls) and not receiving oestradiol benzoate decreased by 40% between 0 and 70 min, and



decreased by a further 50% between 70 min and 150 min. The PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> endometrial synthesizing capacities of control guinea-pigs decreased between 0 min and 70 min but did not decline further between 70 min and 150 min. Interestingly, oestradiol treatment prevented the decrease in endometrial PG synthesizing capacity with time. The initial outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were high during the first 10 min of superfusion, but subsequently, basal outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> remained steady throughout the subsequent period of superfusion (up to 150 min).

Treatment with intra-uterine actinomycin D specifically inhibited endometrial PGF<sub>2α</sub> synthesizing capacity, but had no effect on myometrial PGF<sub>2α</sub> synthesizing capacity, or endometrial or myometrial PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesizing capacities. Oestradiol benzoate treatment did not overcome the inhibitory effect of actinomycin D treatment in vivo on endometrial PGF<sub>2α</sub> synthesizing capacity, and in fact endometrial, but not myometrial, PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesizing capacities were inhibited in actinomycin D-treated guinea-pigs also receiving oestradiol benzoate.

Uterine PGF<sub>2α</sub> output from all uterine horns, irrespective of whether they were not treated or treated with actinomycin D in vivo, and not treated or treated with oestradiol benzoate, was stimulated approximately 3-fold by A23187 treatment. Actinomycin D treatment therefore did not exert any inhibitory effect on this stimulatory action on PG output by A23187. The outputs of 6-keto-PGF<sub>1α</sub> and, to a lesser extent, of PGE<sub>2</sub> from all uteri were also increased by A23187 treatment.

### SECTION 3.5: DISCUSSION

The basal output of  $\text{PGF}_{2\alpha}$  from the superfused guinea-pig uterus in vitro was low when removed on day 7 of the oestrous cycle. However, by day 15,  $\text{PGF}_{2\alpha}$  output had increased approximately 20-fold when compared to  $\text{PGF}_{2\alpha}$  output on day 7. The output of  $\text{PGF}_{2\alpha}$  from the day 7 and day 15 uterus superfused in vitro are similar to those measured by Poyser and Brydon (1983), who demonstrated a 22-fold increase in output between days 7 and 15 of the cycle. Also, the output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium in culture for 24h was 10-15 fold greater than  $\text{PGF}_{2\alpha}$  output from day 7 cultured endometrium.

The basal outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 7 guinea-pig uterus superfused in vitro were similar to day 7  $\text{PGF}_{2\alpha}$  output values. There was little change in  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs on day 15 compared to day 7 of the oestrous cycle from uteri superfused in vitro, and these findings also agree with the results for day 7 and day 15 cultured endometrium. These findings demonstrate a selective stimulation of  $\text{PGF}_{2\alpha}$  output from the guinea-pig endometrium towards the end of the cycle. The injection of intra-uterine saline on day 10 appeared to have no effect on uterine  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from the day 15 uterus superfused in vitro. Also, the outputs of all three PGs from endometrium cultured for 24h removed from animals treated with intra-uterine saline were similar to the corresponding PG outputs over the same time period in culture from day 15 endometrium not treated in vivo on day 10. This demonstrates that the invasive procedure to inject the uterine horns on day 10 had no effect on uterine PG synthesis on day 15.

Actinomycin D, cycloheximide and puromycin inhibited the synthesis of cellular and secreted proteins by day 7 and day 15

guinea-pig endometrium in culture. Furthermore, intra-uterine actinomycin D treatment in vivo on day 10 inhibited the synthesis of cellular and secreted proteins by endometrium removed on day 15 of the oestrous cycle and cultured for 24h. Actinomycin D treatment in vivo on day 10 also inhibited the uterotrophic effect of exogenous oestradiol administered on days 11-14 and also inhibited, but not significantly, the uterotrophic effect of endogenous oestradiol.

The intra-uterine injection of actinomycin D on day 10 caused an 80-85% reduction in  $\text{PGF}_{2\alpha}$  output from the day 15, superfused guinea-pig uterus. Actinomycin D treatment in vivo on day 10 also inhibited  $\text{PGF}_{2\alpha}$  output from endometrium removed on day 15 of the cycle and then cultured for 24h. Plasma progesterone levels in the animals receiving actinomycin D were high on day 15 when compared to the saline-treated, control animals. Clearly, intra-uterine actinomycin D treatment in vivo on day 10 by its inhibitory action on endometrial protein synthesis, inhibits the selective stimulation of  $\text{PGF}_{2\alpha}$  that occurs towards the end of the cycle as demonstrated by the low  $\text{PGF}_{2\alpha}$  output measured on day 15, so removing the luteolytic effect of the uterus, in vivo.

Actinomycin D, cycloheximide and puromycin had an inhibitory effect on both cellular and secreted protein syntheses. However, there were several examples of an inhibition of endometrial PG synthesizing capacity by these compounds occurring before an inhibition of PG output, which demonstrates that although protein and RNA synthesis inhibitors have an inhibitory action on the replenishment of PGH synthase levels in the endometrium by fresh protein synthesis, this is not the rate limiting step in controlling endometrial PG synthesis. Furthermore, in both animals treated with actinomycin D and saline in vivo, the  $\text{Ca}^{2+}$  ionophore A23187 stimulated

PG synthesis approximately three-fold, irrespective of treatment. This shows that the  $\text{Ca}^{2+}$ -induced stimulation of PG synthesis is not limited by uterine PGH synthase concentrations, and this finding is in agreement with results already discussed in section 3.2. Furthermore, protein and RNA synthesis inhibitors superfused over both day 7 and day 15 guinea-pig uteri in vitro had no inhibitory effect on endometrial and myometrial PGH synthase concentrations (measured as PG synthesizing capacity) after a short period (about 1h). This demonstrates that PGH synthase levels in the guinea-pig uterus are not rapidly reduced, unlike findings by Fagan and Goldberg (1986) in rat extensor digitorum longus muscle (a skeletal muscle preparation), where PGH synthase levels in the muscle rapidly (in less than 60 min) declined during treatment with protein synthesis inhibitors. Fagan and Goldberg (1986) proposed that PGH synthase was rapidly used, resulting in inactivation and then rapidly replenished by protein synthesis, and hence was the rate limiting step in PG synthesis in this preparation. Tissue PGH synthase levels are very high in the guinea-pig uterus (see discussion section 3.2). Therefore, from these data, the amount of substrate available for conversion by PGH synthase appears to be the rate limiting step in endometrial PG synthesis in the guinea-pig.

Unexpectedly, protein and RNA synthesis inhibitors also stimulated PG outputs from the guinea-pig uterus. Cycloheximide significantly increased  $\text{PGE}_2$  output from day 7 cultured endometrium during the first 6h culture period. Furthermore, actinomycin D stimulated  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from day 7, and  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from day 15 guinea-pig uterus superfused in vitro. Puromycin stimulated 6-keto- $\text{PGF}_{1\alpha}$  output from both day 7 and day 15 superfused uterine horns. Cycloheximide stimulated the outputs of

PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7, and PGE<sub>2</sub> and 6-keto-PGF<sub>2α</sub> outputs from day 15 superfused uterine horns. Cycloheximide particularly stimulated PGE<sub>2</sub> output from both day 7 and day 15 uteri.

Paradoxically, protein synthesis inhibitors, such as cycloheximide, increase the levels of certain proteins or their corresponding mRNAs. Ringold, Diekmann, Vannice, Trahey and McCormick (1984) in Chinese hamster ovary cells transfected with a plasmid carrying the human  $\beta$ -interferon gene found that cycloheximide, in the absence of any other inducer, stimulated the production of interferon RNA. Sorrentini, Battistini, Curatola and Di Francesco (1985) examined the effect of cycloheximide (0.1 - 50  $\mu$ g/ml) on a number of murine and human cell lines and found a pattern of protein synthesis consisting of enhanced and induced protein species in two main classes of 20-40 and 70-120kD. Using Balb/c 3T3 cells, Hamilton, Nilsen-Hamilton and Adams (1985) demonstrated a 2 to 5-fold increase in production of 'superinducible proteins' (six proteins ranging from 12.5-62kDa). Superinduction occurred at concentrations of cycloheximide (1  $\mu$ g/ml) which inhibited protein synthesis by at least 85%, and the greatest amounts of these proteins were observed after 6-8h. From these findings it may be speculated that the protein and RNA synthesis inhibitors, particularly cycloheximide, are stimulating a protein that stimulates uterine PG synthesis, especially PGE<sub>2</sub> output during the first 6h of culture from day 7 endometrium. Alternatively, it may be argued that the effects of protein and RNA synthesis inhibitors on PG outputs from superfused uterine horns are very rapid, with insufficient time for a stimulatory protein to be induced. The effect of the inhibitors may be therefore a non-specific effect, for instance on the membrane causing the release of more substrate which produces a transitory increase in PG output. As

the stimulation of PGE<sub>2</sub> output by cycloheximide is the largest observed, the apparent stimulation of PGE<sub>2</sub> observed in culture may be due to this non-specific unspecified effect causing a stimulation of PGE<sub>2</sub> output early in the initial 6h culture period, which may mask the inhibitory action of cycloheximide on PGE<sub>2</sub> output later in the first sample period, as observed in subsequent sample periods.

Steroids have been shown to act via the stimulation of protein synthesis in the uterus (Brenner and West, 1975). Oestradiol acting on a progesterone-primed uterus is the optimum stimulus for the increase in PGF<sub>2α</sub> output that occurs towards the end of the oestrous cycle in the guinea-pig (Poyser, 1983b). In this section, protein and RNA synthesis inhibitors have been demonstrated to inhibit both protein synthesis and PG synthesis. These findings indicate that the stimulatory effect of steroid hormones on uterine PG synthesis is due to their effect on increasing protein synthesis in the uterus. Therefore, the next series of experiments (section 3.6) addresses this question, and examines the role of oestradiol, progesterone, and other hormones that may have an action on both PG production and on protein synthesis by the guinea-pig endometrium.

### 3.6 EFFECTS OF OESTRADIOL, PROGESTERONE, HYDROCORTISONE AND OXYTOCIN ON PROSTAGLANDIN PRODUCTION BY THE GUINEA-PIG ENDOMETRIUM IN CULTURE

#### INTRODUCTION

In ovariectomised guinea-pigs, the action of oestradiol on a progesterone-primed uterus causes a large stimulation of  $\text{PGF}_{2\alpha}$  output (Blatchley and Poyser, 1974). In intact animals, oestradiol output from the guinea-pig ovary has been shown to increase after day 10 of the cycle (Joshi, Watson and Labhsetwar, 1973) and, after day 11,  $\text{PGF}_{2\alpha}$  output increases from the uterus (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975; Antonini, Turner and Pauerstein, 1976). Therefore, it has been proposed that these ovarian steroid hormones are the physiological stimulus for endometrial  $\text{PGF}_{2\alpha}$  production (Poyser, 1983b). However, progesterone has been shown to inhibit basal and oestradiol stimulated  $\text{PGF}_{2\alpha}$  output from the human endometrium and the guinea-pig endometrium maintained in tissue culture (Cane and Villee, 1975; Abel and Baird, 1980; Leaver and Seawright, 1982), and also from human endometrial cells maintained in culture (Schatz, Markiewicz, Barg and Gurside, 1985). In vivo, plasma progesterone concentrations are required to fall in several mammalian species before maximal  $\text{PGF}_{2\alpha}$  output can occur (Horton and Poyser, 1976).

Glucocorticoid hormones exert part of their anti-inflammatory action by increasing the production of lipocortins, which inhibit phospholipase (PL)  $A_2$  activity and so reduce the amount of arachidonic acid released from phospholipids and which is available for prostaglandin, thromboxane and leukotriene production (Blackwell and Flower, 1983). Recently, lipocortins have been implicated in the control of PG synthesis by the human endometrium (Gurside, Markiewicz,



Schatz and Hirata, 1986), since there was a clear relationship between increased lipocortin and decreased PG levels found in the culture medium.

This study has examined the effects of steroid hormones and of oxytocin on PG synthesis by the guinea-pig uterus by measuring PG outputs from and PG synthesizing capacities of guinea-pig endometrium in tissue culture. The effects of ovarian steroids on fresh protein synthesis are also examined.

The aims of this study are:

- (a) to investigate whether hydrocortisone, the main glucocorticoid hormone secreted in the guinea-pig (Fajer and Vogt, 1963), has an inhibitory effect on  $\text{PGF}_{2\alpha}$  output from guinea-pig endometrium.
- (b) to investigate whether the inhibitory effect of progesterone on endometrial  $\text{PGF}_{2\alpha}$  production is due to a glucocorticoid-like action.
- (c) to confirm a previous report by Leaver and Seawright (1982) that oestradiol and oxytocin stimulate  $\text{PGF}_{2\alpha}$  output from cultured guinea-pig endometrium.
- (d) to examine the effects of oestradiol, progesterone, hydrocortisone and oxytocin on the outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig endometrium.
- (e) to investigate the actions of oestradiol and progesterone on the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  produced by guinea-pig endometrial homogenates after culture.
- (f) to examine whether the effects of oestradiol and progesterone on endometrial  $\text{PGF}_{2\alpha}$  production are related to hormone-induced changes in protein synthesis.

## METHODS

Female guinea-pigs were used on either day 7 or day 15 of the



oestrous cycle and their uteri were removed in preparation for tissue culture as detailed in section 2.2.1. Endometrium was separated from the myometrium and small pieces of endometrium were placed in petri dishes according to the method for tissue culture (section 2.2.5). In Experiments 1, 2 and 3, each dish contained between 12 and 20mg endometrium, and in Experiment 4, between 30 and 60mg endometrium. The dishes were placed in modified Kilner jars, which were gassed and incubated under the conditions detailed in section 2.2.5. Details of the individual experimental procedures are given below. All culture medium and ethyl acetate samples were stored at  $-20^{\circ}\text{C}$  before the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present were measured by radioimmunoassay (see sections 2.3.1, 2.3.2, 2.3.3. and 2.3.4).

#### Experiment 1

Day 7 and day 15 guinea-pigs were used (5 animals per day). Endometrium removed from the uterus of each animal was placed into eighteen Petri dishes in preparation for tissue culture as explained above. These dishes were divided equally between two Kilner jars (i.e. nine dishes/jar), which were gassed and incubated for 24h as detailed above.

After 24h, the culture medium was changed. Two of the Petri dishes in each jar were left untreated (controls), and each hormone concentration was added to one of the remaining dishes from each jar (i.e. each treatment was performed in duplicate with one of each of the duplicate treatments being placed in each jar). The treatments are specified below. The dishes were replaced in the jars, which were gassed and incubated (as above) for a further 24h.

After 48h of culture the medium was changed again, but no further treatment was administered. The dishes were replaced in the jars, which were gassed and incubated (as above) for a further 24h.

After 72h of culture, the culture medium was collected and the endometrium in each dish was weighed.

Treatments: Endometrium on day 7 and day 15 of the oestrous cycle was treated on day 2 (between 24h and 48h) with oestradiol-17 $\beta$  (10,100 and 1,000ng/ml), progesterone (10 and 100ng/ml) and oxytocin ( $10^{-5}$  and  $10^{-4}$ U/ml).

#### Experiment 2

The procedure for this experiment was very similar to that of Experiment 1. Uteri were removed from five day 7 and five day 15 guinea-pigs. Endometrium from each uterus was placed into eighteen Petri dishes in preparation for tissue culture. The dishes were divided equally between two Kilner jars. Each jar contained two control dishes and one dish of each treatment performed in duplicate as specified below. The jars were gassed and incubated for 24h. The medium was changed every 24h, as in Experiment 1. However, endometrium in these Petri dishes was treated on all three days of culture (the hormone treatment was added at time 0h and after each change of medium at 24h and 48h) as specified below:

Treatments: Day 7 endometrium was treated on all three days of culture with oestradiol-17 $\beta$  (1, 10, 100 and 1000ng/ml). Day 15 endometrium was treated on all three days of culture with oestradiol-17 $\beta$  (1, 10, 100 and 1000ng/ml), progesterone (1, 10, 100 and 1000ng/ml), hydrocortisone (1, 10, 100 and 1000ng/ml) or oxytocin ( $10^{-6}$ ,  $10^{-5}$  and  $10^{-4}$ U/ml).

#### Experiment 3

Uteri from six guinea-pigs on day 7 and from six guinea-pigs on day 15 of the oestrous cycle were removed in preparation for tissue culture as above. Endometrium was dissected from each uterus and was placed into twenty Petri dishes, according to the tissue culture

methods as before. These dishes were divided into five groups of four dishes and each group was treated with one of the following: oestradiol-17 $\beta$  (10ng/ml); oestradiol-17 $\beta$  (1000ng/ml); progesterone (10ng/ml); progesterone (100ng/ml); the final group remained untreated (controls). The groups of dishes were distributed between three Kilner jars, so that in two jars there were four groups (i.e. two groups/jar), with the final group in the third jar. The jars were gassed and incubated as specified above.

Immediately after the jars were placed in the incubator, between 12-20mg of the remaining endometrium was weighed accurately, homogenised and incubated for 60 min with 2 $\mu$ g sodium arachidonate (added in 10 $\mu$ l ethanol) according to the procedure and conditions explained in section 2.2.4.ii. This homogenate measured the "0h" control PG synthesizing capacity of the endometrium. After incubation, PGs were extracted from the incubate and redissolved in 5ml ethyl acetate as explained in section 2.2.2.

After 6h, 12h and 18h of culture, one dish was removed from each group of dishes at each time. The medium from the remaining dishes was collected and replaced with both fresh medium and the appropriate steroid treatment. The dishes were replaced in the jars, which were gassed and incubated as specified above. After 24h of culture, the remaining five dishes were removed.

Immediately after removal, the culture medium from the dishes was collected. The tissue was weighed, homogenised and incubated for 60 min with 2 $\mu$ g sodium arachidonate (section 2.2.4.ii), and the PGs were then extracted (section 2.2.2) as explained for "0h" controls above. These homogenates measured endometrial PG synthesizing capacities after 6h, 12h, 18h and 24h of culture.

#### Experiment 4

Five day 7 and five day 15 guinea-pigs were killed and their uteri removed (as above). Endometrium was dissected from each uterus and was placed in five Petri dishes in preparation for tissue culture as specified above (30-60mg tissue/dish). Each dish also contained 10  $\mu\text{Ci}$  [ $^3\text{H}$ ]-Leucine (2.5 $\mu\text{Ci}/\text{ml}$ ), consistent with the method for measuring protein synthesis (section 2.4). These dishes were placed in a modified Kilner jar, which was gassed and incubated for 24h as detailed above. Immediately after the jar was placed in the incubator, 30-60mg of the remaining endometrium was placed in another Petri dish, according to the tissue culture method, which contained 10 $\mu\text{Ci}$  [ $^3\text{H}$ ]-leucine (2.5 $\mu\text{Ci}/\text{ml}$ ) in the medium. The culture medium was washed over the endometrium using a Pasteur pipette and the dish was incubated for 2 min at room temperature. The endometrium was then weighed accurately. The endometrium and culture medium from this dish measured the non-specific binding of [ $^3\text{H}$ ]-leucine that occurred during the procedures used to measure [ $^3\text{H}$ ]-leucine incorporation into freshly synthesised cellular and secreted proteins, respectively (section 2.4). After 24h of culture, the incubated dishes were removed, and the incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted proteins was measured by analysing the endometrium and culture medium, respectively, as specified in section 2.4.

#### Presentation of results, calculations and statistical analysis

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig endometrium in Experiments 1 and 2 are expressed as a percentage of their respective day 1 control output value for the same set of dishes. The control output values in Experiment 1 are the day 1 values obtained from every dish for each PG. The control output values in Experiment 2 are the mean of the two values obtained for the

untreated dishes in each jar for each PG. There was insufficient endometrium to test all the hormones at all the concentrations using endometrium removed from the same guinea-pig. By exhibiting the results in this form, the effects of different hormone treatments on the outputs of PGs from endometrium removed from different animals can be compared.

Changes with time in PG outputs from and PG synthesizing capacities of endometrium in Experiment 3 were examined using Duncan's multiple range test. Other comparisons were made using Student's 't' test (or the modified 't' test if variances were unequal by the variance F-test) in Experiments 1, 2, 3 and 4, as appropriate.

In Experiment 4, the non-specific binding of [ $^3\text{H}$ ]-leucine at time 0h that occurred in the endometrium and culture medium was subtracted from the amount of [ $^3\text{H}$ ]-leucine incorporated into cellular and secreted proteins, respectively, after 24h in culture.

## RESULTS

Basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from endometrium removed on day 7 and day 15 of the oestrous cycle and cultured for three days are shown in Figure 3.1.a in Section 3.1. Briefly, when day 7 endometrium is cultured for three days,  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs are similar, while  $\text{PGE}_2$  output is lower. When day 15 endometrium is cultured for three days,  $\text{PGF}_{2\alpha}$  is the main PG released, with lesser amounts of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  being released. Outputs of all PGs from both day 7 and day 15 endometrium declined during the three day culture period (see section 3.1).

### Experiment 1

Oestradiol (10, 100 and 1000ng/ml) treatment of day 7 and day 15 endometrium on day 2 of culture had no stimulatory action on the

outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  either on day 2 (during treatment) or on day 3 (after treatment; Figures 3.6.a and 3.6.b). In fact, oestradiol (1000ng/ml) treatment on day 2 of culture significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from day 7, and  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs from day 15 endometrium on day 3 of culture (Figure 3.6.a and 3.6.b). Progesterone (100ng/ml) treatment on day 2 of culture significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs on day 2 of culture from day 7 endometrium, and significantly ( $P < 0.05$ ) inhibited the output of  $\text{PGE}_2$  on day 2, and the output of  $\text{PGF}_{2\alpha}$  on day 3 of culture from day 15 endometrium. Also, progesterone (10ng/ml) treatment significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  output from day 7 endometrium on day 2 of culture. The output of 6-keto- $\text{PGF}_{1\alpha}$  was not changed by progesterone treatment from either day 7 or day 15 endometrium (Figures 3.6.a and 3.6.b respectively). Oxytocin ( $10^{-5}$  and  $10^{-4}\text{U/ml}$ ) treatment had no effect on the output of any PG from either day 7 or day 15 endometrium on either day 2 or day 3 of culture (Figures 3.6.a and 3.6.b, respectively).

## Experiment 2

Oestradiol treatment (1, 10 and 100ng/ml) had no effect on  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs on any day of culture, from either day 7 or day 15 endometrium (Figures 3.6.c and 3.6.d, respectively). Addition of oestradiol (1000ng/ml) significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  output on all three days of culture from day 7 and day 15 endometrium. Oestradiol (1000ng/ml) treatment also significantly ( $P < 0.05$ ) reduced  $\text{PGE}_2$  outputs on days 1 and 2 of culture from day 7 endometrium, and on days 2 and 3 from day 15 endometrium in culture. Oestradiol-treated endometrium from either day 7 or day 15 of the oestrous cycle exhibited no significant change in 6-keto- $\text{PGF}_{1\alpha}$  output on any day of

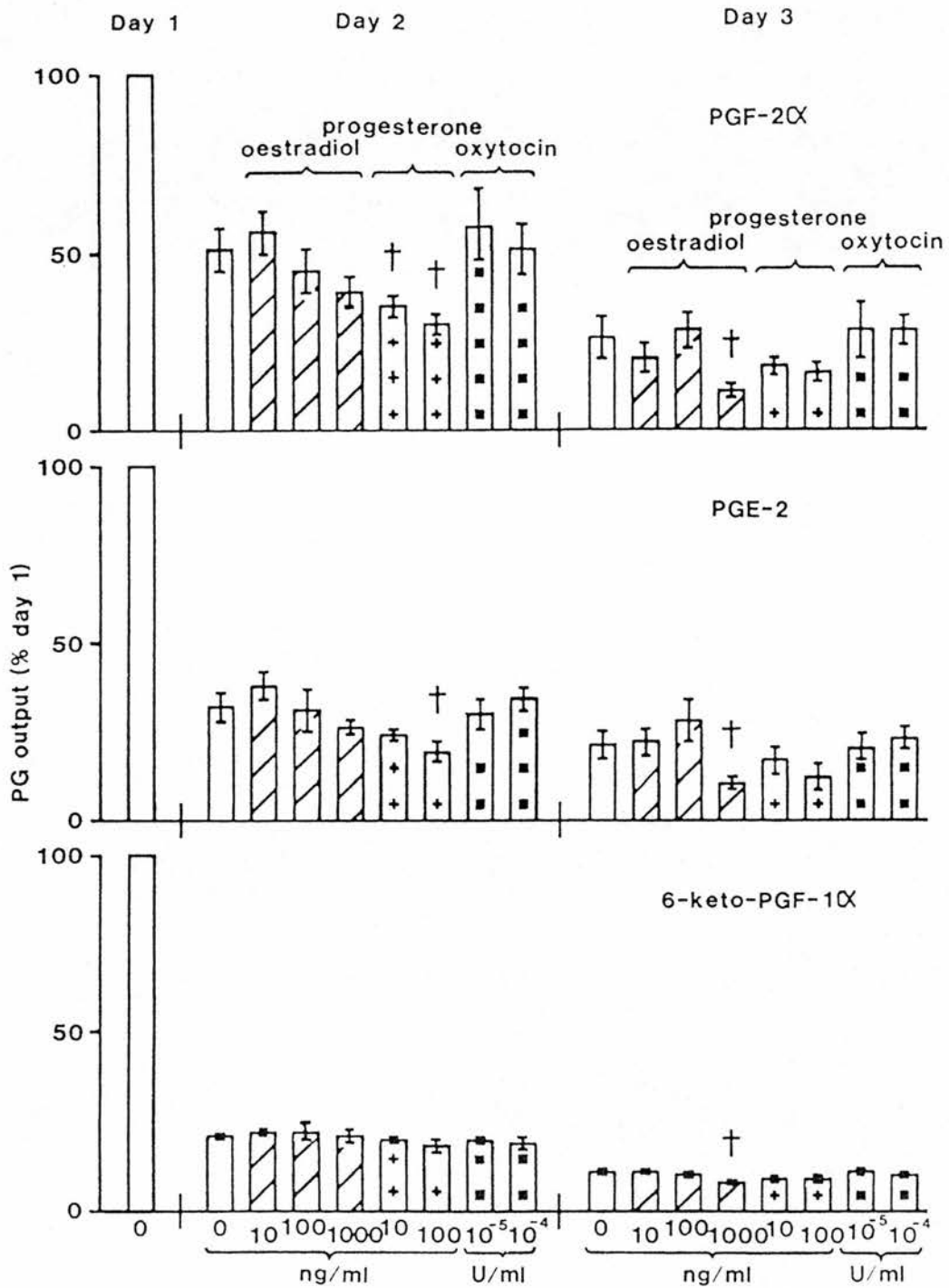


Figure 3.6.a

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  (expressed as a percentage of day 1 PG output value) from day 7 guinea-pig endometrium in culture for three days, sampling every 24h and not treated (controls (0); open columns) or treated with oestradiol (10, 100 and 1000ng/ml; hatched columns), progesterone (10 and 100ng/ml; columns with crosses) and oxytocin (10<sup>-5</sup> and 10<sup>-4</sup>U/ml; columns with squares) on day 2 of culture.

† significantly ( $P < 0.05$ ) lower than the corresponding control value for the same PG on the same day of culture.

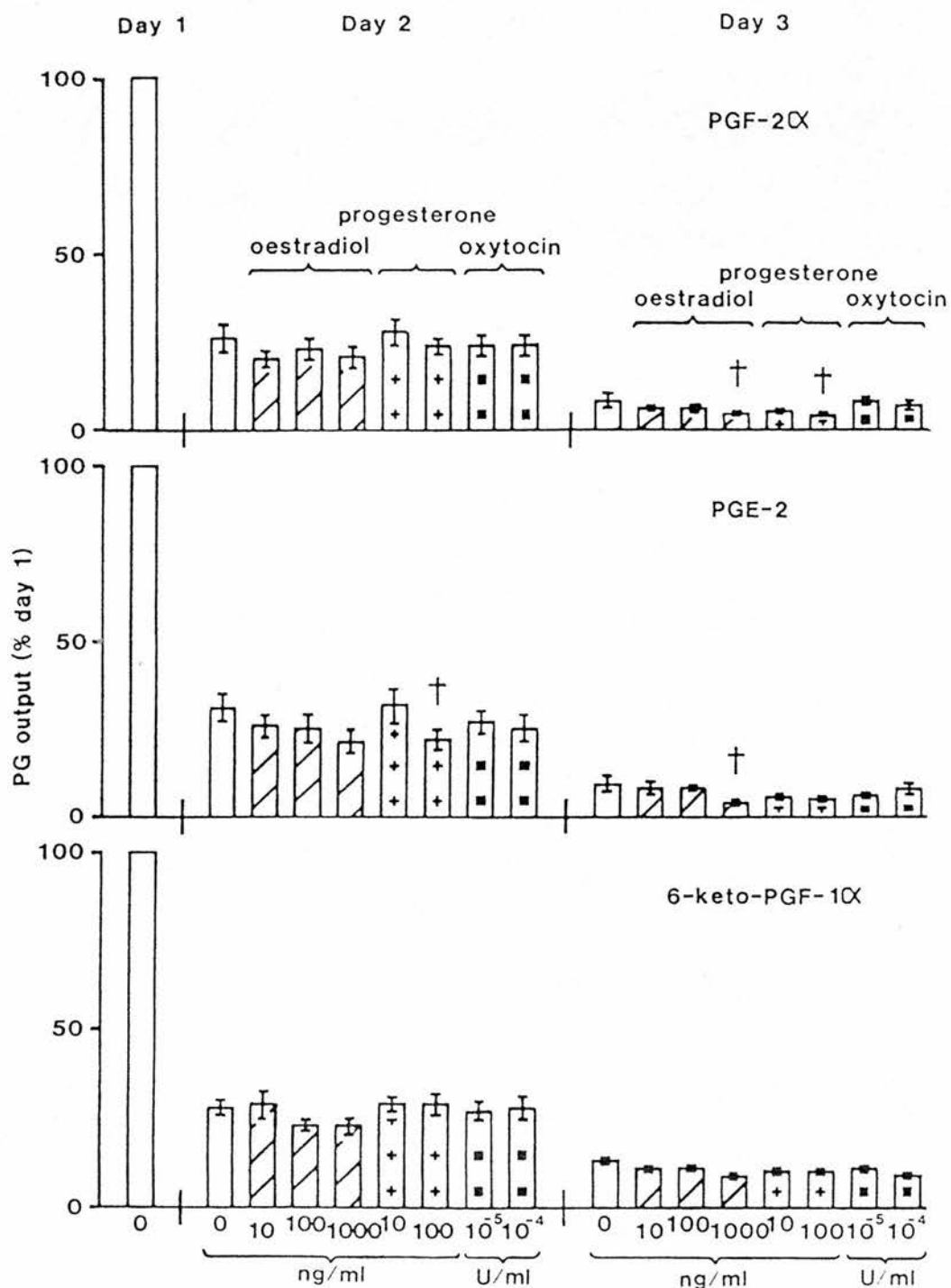


Figure 3.6.b

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  (expressed as a percentage of day 1 PG output value) from day 15 guinea-pig endometrium in culture for three days, sampling every day, and not treated (controls (0); open columns) or treated with oestradiol (10, 100 and 1000ng/ml; hatched columns), progesterone (10 and 100ng/ml; columns with crosses) and oxytocin (10<sup>-5</sup> and 10<sup>-4</sup>U/ml; columns with squares) on day 2 of culture.

† significantly ( $P < 0.05$ ) lower than the corresponding control value for the same PG on the same day of culture.



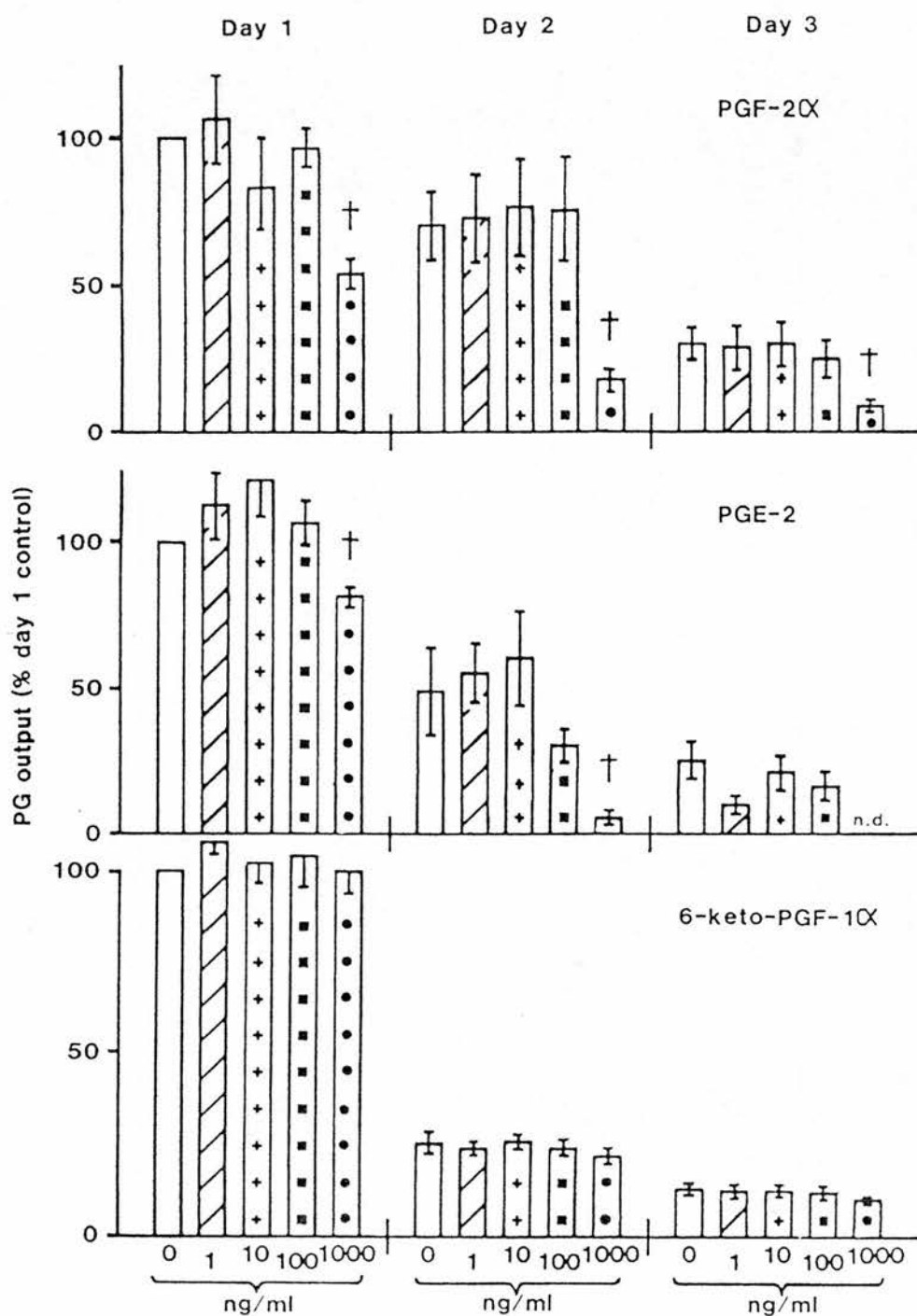


Figure 3.6.c

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  (expressed as a percentage of day 1 control (0) value) from day 7 cultured guinea-pig endometrium and not treated (control (0); open columns) or treated with  $1\text{ng/ml}$  (hatched columns),  $10\text{ng/ml}$  (columns with crosses),  $100\text{ng/ml}$  (columns with squares) and  $1000\text{ng/ml}$  (columns with circles) oestradiol on all three days of culture.  
† significantly ( $P < 0.05$ ) lower than the corresponding control (0) value for the same PG on the same day of culture.  
n.d. below the detection level for  $\text{PGE}_2$ .

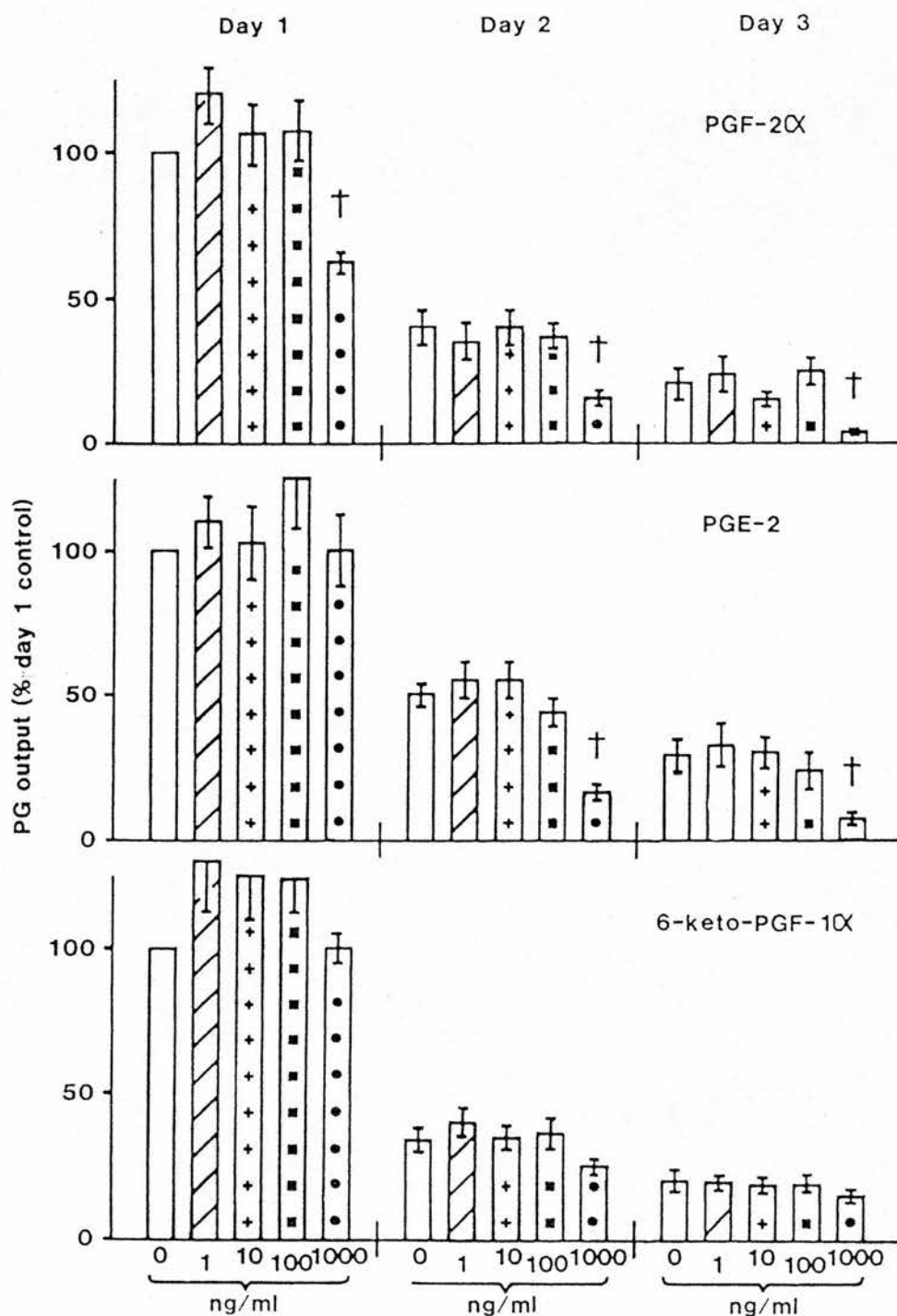


Figure 3.6.d

Mean (± s.e.m.;  $n = 10$ ) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α (expressed as a percentage of day 1 control (0) value) from day 15 cultured guinea-pig endometrium and not treated (control (0); open columns) or treated with 1ng/ml (hatched columns), 10ng/ml (columns with crosses), 100ng/ml (columns with squares) and 1000ng/ml (columns with circles) oestradiol on all three days of culture.

† significantly ( $P < 0.05$ ) lower than the corresponding control (0) value for the same PG on the same day of culture.

culture compared to the control values on the same day of culture (Figures 3.6.c and 3.6.d).

Treatment of day 15 endometrium with oxytocin ( $10^{-6}$ ,  $10^{-5}$  and  $10^{-4}$  U/ml) had no significant effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$  on any day of culture (Figure 3.6.e).

Progesterone (1, 10, 100 and 1000ng/ml) treatment significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  output from day 15 endometrium on days 1 and 2 of culture, except progesterone (1ng/ml) treatment had no significant effect on day 1 of culture (Figure 3.6.f). Progesterone (1000ng/ml) treatment significantly ( $P < 0.05$ ) inhibited  $\text{PGF}_{2\alpha}$  output from day 15 endometrium on day 3 of culture, but lower concentrations of progesterone exhibited no action on  $\text{PGF}_{2\alpha}$  output on day 3 of culture (Figure 3.6.f). The output of  $\text{PGE}_2$  was not changed by progesterone treatment, except  $\text{PGE}_2$  output was significantly ( $P < 0.05$ ) inhibited by progesterone (100ng/ml) on day 2, and by progesterone (1000ng/ml) on days 1 and 2 of culture. Progesterone treatment had no significant effect on 6-keto- $\text{PGF}_{1\alpha}$  output from day 15 endometrium on any day of culture (Figure 3.6.f).

Hydrocortisone (1, 10, 100 and 1000ng/ml) treatment had no significant effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium in culture for three days (Figure 3.6.g).

### Experiment 3

The effects of oestradiol and progesterone on PG output from and PG synthesizing capacity of guinea-pig endometrium maintained in culture for up to 24h, are shown in Figures 3.6.h to 3.6.o. The control (C) results have been presented twice in both sets of steroid results for clarity, and they will be examined first in this results section.

Control values: The output of  $\text{PGF}_{2\alpha}$  from day 7 endometrium between 0

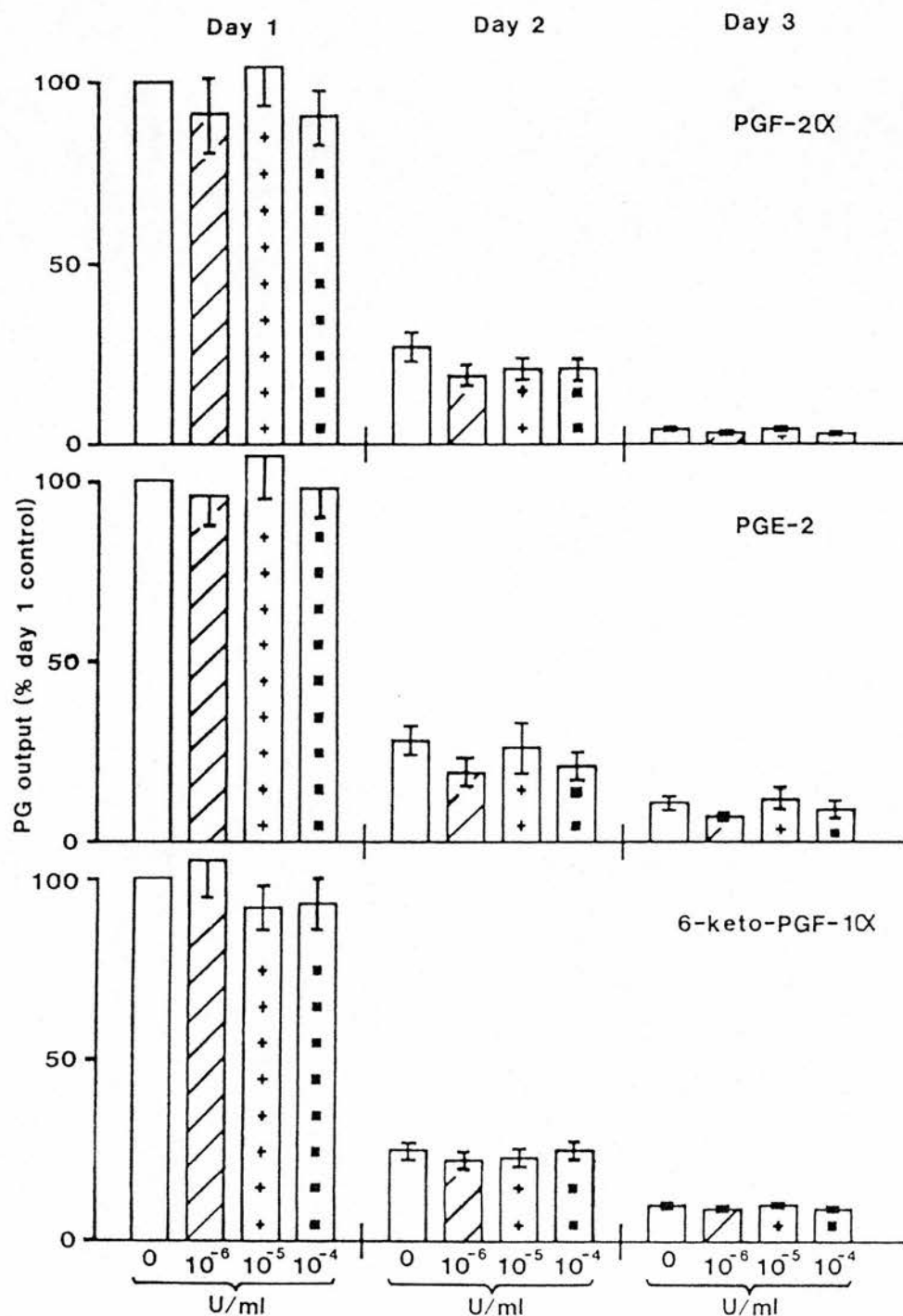


Figure 3.6.e

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of PGF<sub>2</sub> $\alpha$ , PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  (expressed as a percentage of day 1 control (0) value) from day 15 cultured guinea-pig endometrium and not treated (control (0); open columns) or treated with 10<sup>-6</sup>U/ml (hatched columns), 10<sup>-5</sup>U/ml (columns with crosses) and 10<sup>-4</sup>U/ml (columns with squares) oxytocin on all three days of culture.

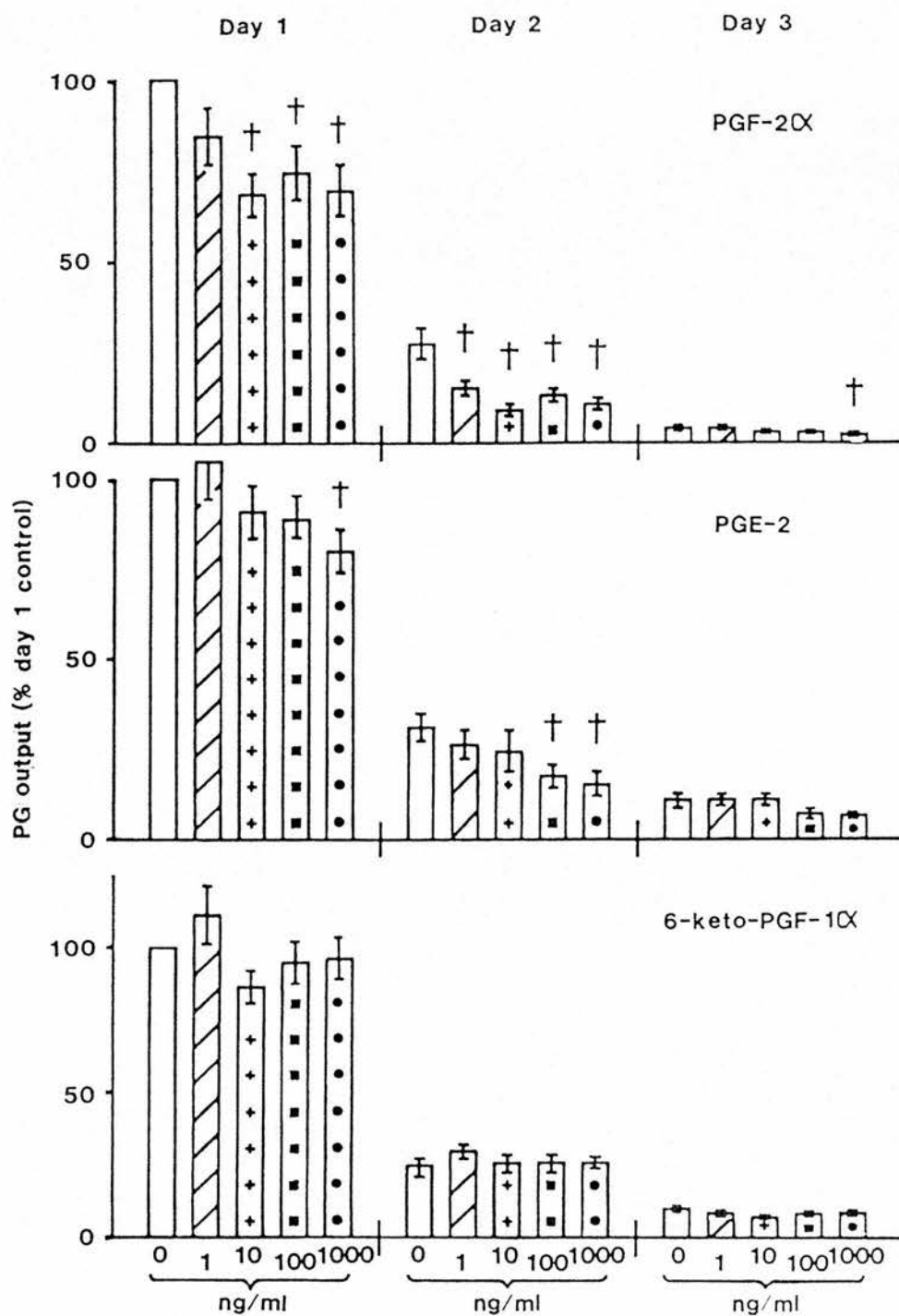


Figure 3.6.f

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (expressed as a percentage of day 1 control (0) value) from day 15 cultured guinea-pig endometrium and not treated (control (0); open columns) or treated with 1ng/ml (hatched columns), 10ng/ml (columns with crosses), 100ng/ml (columns with squares) and 1000ng/ml (columns with circles) progesterone on all three days of culture.

† significantly ( $P < 0.05$ ) lower than the corresponding control (0) value for the same PG on the same day of culture.

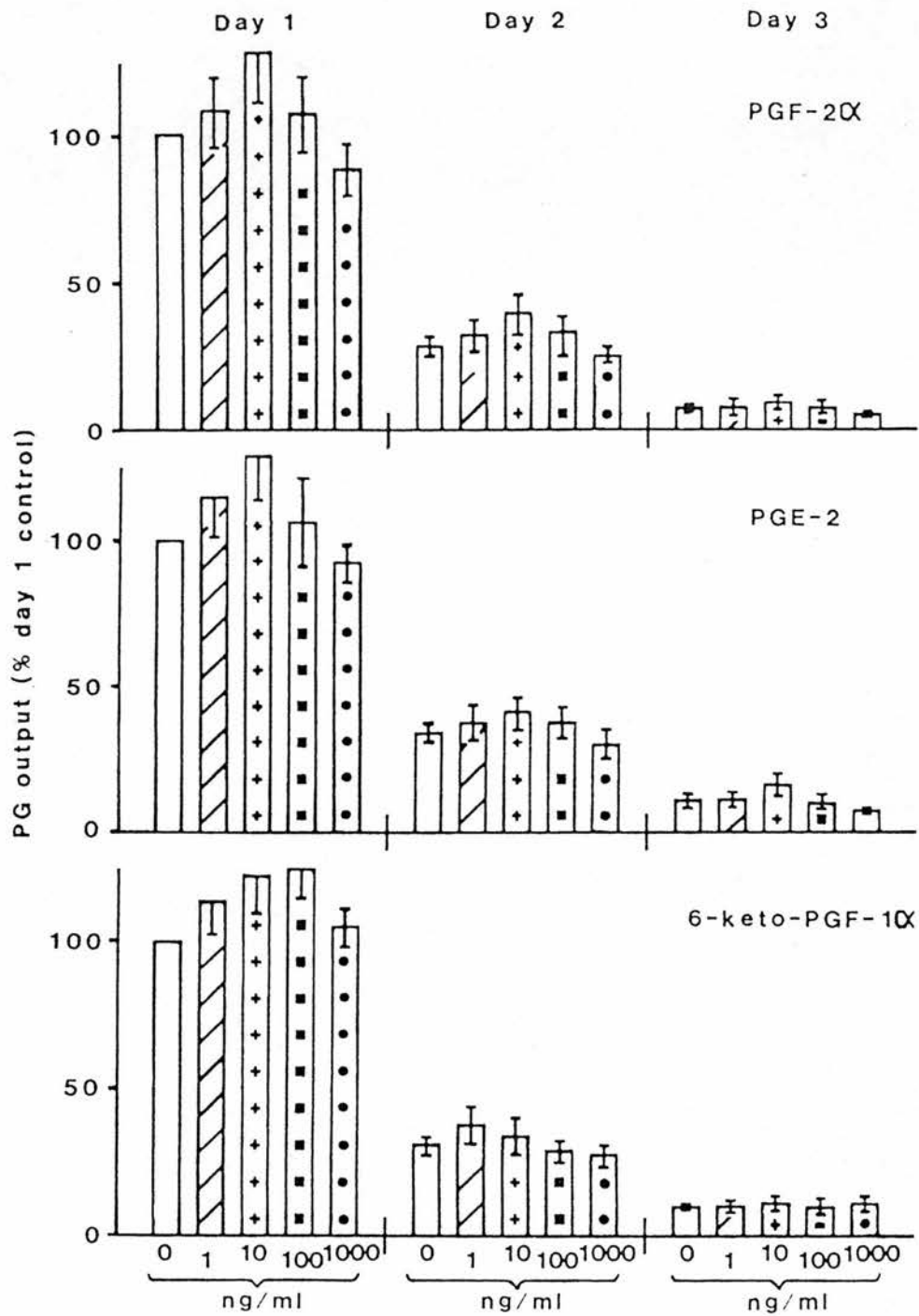


Figure 3.6.g

Mean ( $\pm$  s.e.m.;  $n = 10$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (expressed as a percentage of day 1 control (0) value) from day 15 cultured guinea-pig endometrium and not treated (control (0); open columns) or treated with 1ng/ml (hatched columns), 10ng/ml (columns with crosses), 100ng/ml (columns with squares) and 1000ng/ml (columns with circles) hydrocortisone on all three days of culture.

† significantly ( $P < 0.05$ ) lower than the corresponding control (0) value for the same PG on the same day of culture.

and 6h decreased slightly, but not significantly, to the lowest output which was recorded between 6h and 12h (Figures 3.6.h and 3.6.l). The  $\text{PGF}_{2\alpha}$  output then increased between 12h and 18h and was significantly ( $P < 0.05$ ) greater than the  $\text{PGF}_{2\alpha}$  output between 6h and 12h. This significantly ( $P < 0.05$ ) higher output of  $\text{PGF}_{2\alpha}$  was maintained until the end of the 24h culture period. The output of  $\text{PGE}_2$  declined significantly ( $P < 0.05$ ) from its highest level between 0 and 6h to a lower level of output between 6h and 12h, and this significantly ( $P < 0.05$ ) lower output of  $\text{PGE}_2$  remained constant for the remainder of the culture period (Figures 3.6.h and 3.6.l). The output of 6-keto- $\text{PGF}_{1\alpha}$  from day 7 endometrium declined significantly ( $P < 0.05$ ) throughout the culture period (Figures 3.6.g and 3.6.l).

The amounts of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  synthesised by homogenates of day 7 endometrium following culture for up to 24h significantly ( $P < 0.05$ ) increased from the 0h control value up to the 18h sample time for  $\text{PGF}_{2\alpha}$ , and up to the 12h sample time for  $\text{PGE}_2$  (Figures 3.6.i and 3.6.m). These increased levels in PG synthesizing capacities were maintained up to the end of the culture period. The synthesizing capacity of 6-keto- $\text{PGF}_{1\alpha}$  of day 7 endometrium did not change significantly from the 0h control value at any of the sample times after culture (Figures 3.6.i and 3.6.m).

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium cultured for up to 24h all significantly ( $P < 0.05$ ) declined during the culture period (Figures 3.6.j and 3.6.m). The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesised by homogenates of day 15 endometrium cultured for up to 24h significantly ( $P < 0.05$ ) increased after 6h of culture, when compared to 0h values (Figure 3.6.k and 3.6.o). The synthesizing capacities of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were significantly ( $P < 0.05$ ) at their highest values after 6h

and 12h of culture, and then they declined from this level after 18h and 24h of culture. However, after 24h of culture, the synthesizing capacities of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were still significantly ( $P < 0.05$ ) higher than their respective 0h control synthesizing capacities. The synthesizing capacity of  $\text{PGE}_2$  was significantly ( $P < 0.05$ ) higher after 6h of culture when compared to the 0h control value; this increased synthesizing capacity for  $\text{PGE}_2$  did not change significantly for the remaining sampling periods (Figures 3.6.k and 3.6.o).

Oestradiol treatment: The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 endometrium were not changed by oestradiol (10ng/ml; Figure 3.6.h). However, the output of  $\text{PGF}_{2\alpha}$  after treatment with oestradiol (1000ng/ml) was significantly ( $P < 0.05$ ) inhibited between 12h and 18h, and 18h and 24h of culture. Furthermore, the rise in control values seen during these periods were prevented by oestradiol (1000ng/ml; Figure 3.6.h). The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were not affected by treatment with oestradiol (1000ng/ml; Figure 3.6.h).

The  $\text{PGF}_{2\alpha}$  synthesizing capacity of day 7 endometrium treated with oestradiol (both concentrations) was significantly ( $P < 0.05$ ) higher after 12h, 18h and 24h of culture when compared to 0h control values (Figure 3.6.i). These increases are the same as exhibited by control (non-treated) endometrium over the same time periods. Furthermore, treatment of endometrium with 10ng oestradiol/ml significantly ( $P < 0.05$ ) increased the  $\text{PGF}_{2\alpha}$  synthesizing capacity after 18h of culture compared to the control value at the same time. Treatment of endometrium with oestradiol (1000ng/ml) after 18h of culture significantly ( $P < 0.05$ ) prevented to some extent the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity exhibited by the control value at the same time, although the oestradiol (1000ng/ml) treated output was



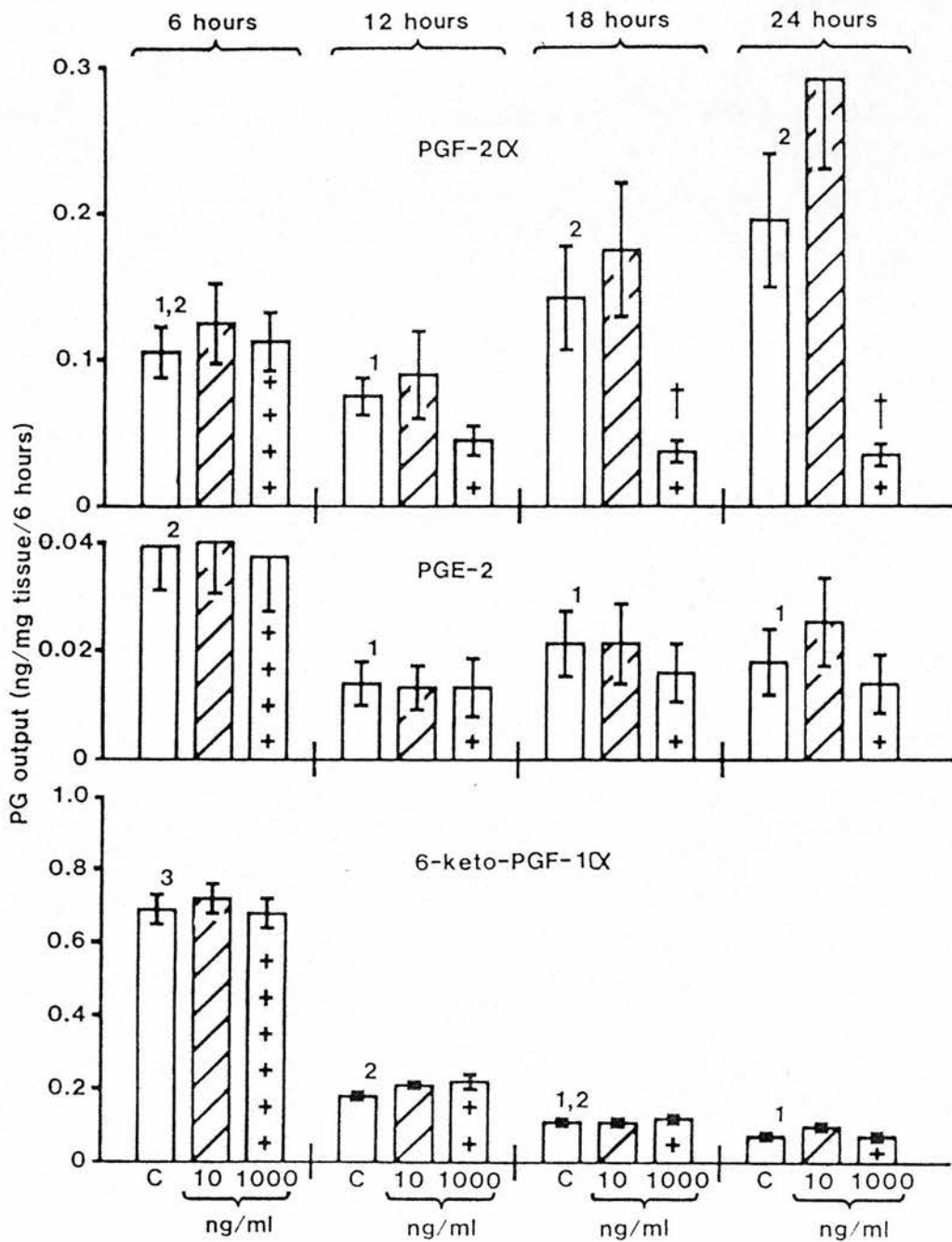


Figure 3.6.h

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  (ng/mg tissue/6h) from guinea-pig endometrium removed on day 7 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h, not treated (controls (C); open columns), or treated with 10ng oestradiol/ml (hatched columns), or 1000ng oestradiol/ml (crossed columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG output at the same time for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

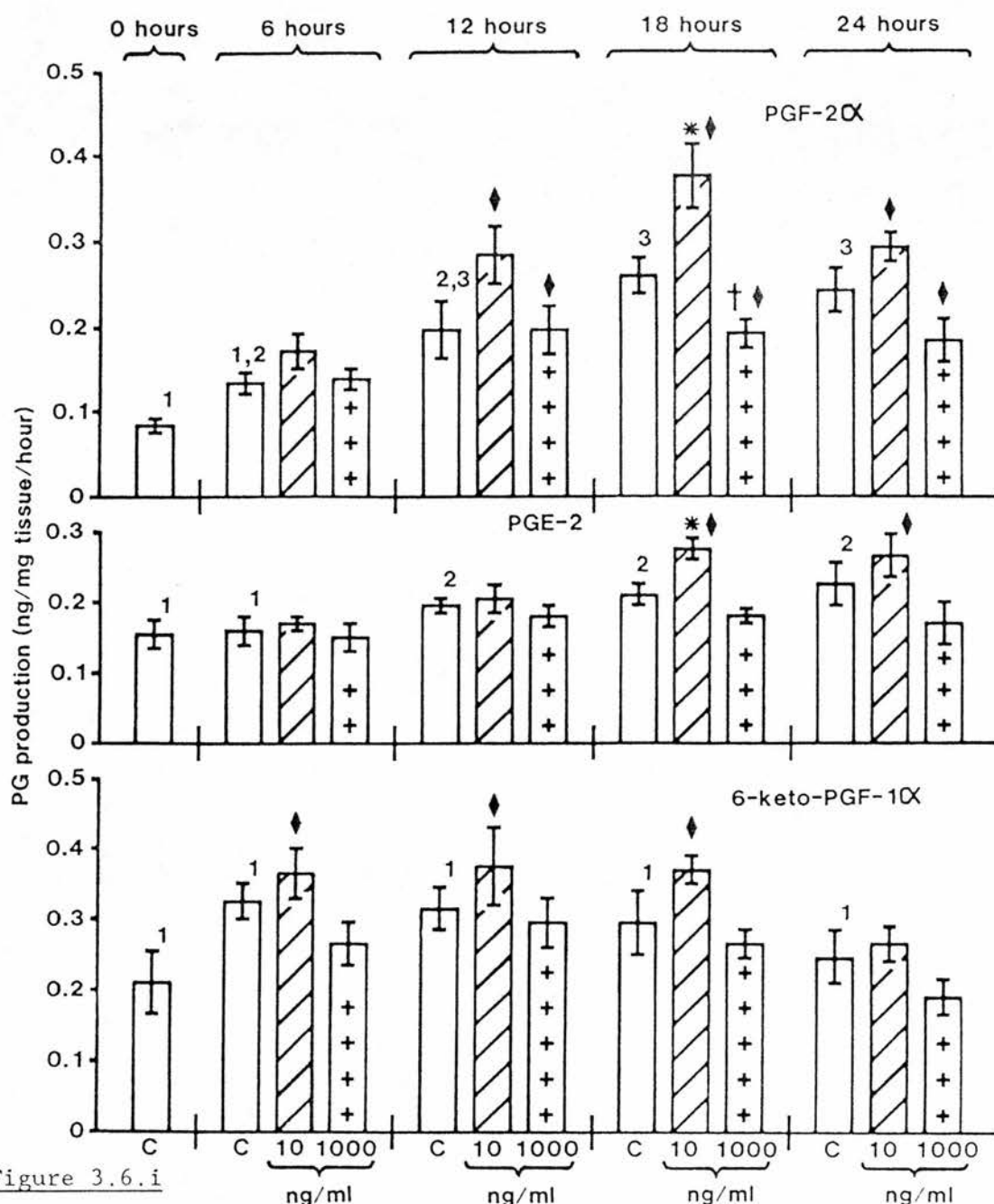


Figure 3.6.i

Mean (± s.e.m.; n = 6) amounts of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α (ng/mg tissue/h) synthesised by homogenates of day 7 guinea-pig endometrium at 0h and after 6h, 12h, 18h and 24h of culture, not treated (control (C); open columns), or treated with 10ng oestradiol/ml (hatched columns) or 1000ng oestradiol/ml (crossed columns) during the culture period.

† significantly (P < 0.05) lower than the control (C) PG production at the same time for the same PG.

♦ significantly (P < 0.05) higher than the 0h control (C) PG production for the same PG.

\* significantly (P < 0.05) higher than control (C) PG production at the same time for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly (P < 0.05) different.

significantly ( $P < 0.05$ ) higher than the 0h control. The  $\text{PGE}_2$  synthesizing capacity of day 7 guinea-pig endometrium was significantly ( $P < 0.05$ ) higher after 18h and 24h of culture than at 0h following treatment with 10ng oestradiol/ml (Figure 3.6.i). This increase in  $\text{PGE}_2$  synthesizing capacity following oestradiol (10ng/ml) treatment was similar to the control value profile of  $\text{PGE}_2$  synthesizing capacity, except that control values increased to a significantly ( $P < 0.05$ ) higher level than 0h controls after 12h of culture, a sample period earlier than the 10ng oestradiol/ml treated endometrial value (Figure 3.6.i). After 18h of culture, 10ng oestradiol/ml treatment significantly ( $P < 0.05$ ) increased  $\text{PGE}_2$  synthesizing capacity to a value greater than the control value at the same time. Oestradiol (1000ng/ml) treatment prevented the increase in  $\text{PGE}_2$  synthesizing capacity observed during culture, so the  $\text{PGE}_2$  synthesizing capacity did not change from the 0h control value. The profile of the amounts of 6-keto- $\text{PGF}_{1\alpha}$  produced by homogenates of day 7 endometrium following 6h to 24h in culture when treated with oestradiol was similar to the control profile of 6-keto- $\text{PGF}_{1\alpha}$  production by homogenates (Figure 3.6.i). However, the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 7 endometrium treated with oestradiol (10ng/ml) was significantly ( $P < 0.05$ ) higher after 6h, 12h and 18h of culture compared to the control value at 0h, although these values were not significantly different from the control values at the same respective times. Oestradiol (1000ng/ml) did not change the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 7 endometrium during culture when compared to the 0h control value and to the control values at the same respective times (Figure 3.6.i).

Treatment with oestradiol (10ng/ml) had no effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium during 24h in

culture (Figure 3.6.j). Oestradiol (1000ng/ml) treatment did not change PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs during 24h of culture, and PGF<sub>2α</sub> output between 0h and 6h and 6h and 12h of culture. However, oestradiol (1000ng/ml) treatment significantly ( $P < 0.05$ ) reduced PGF<sub>2α</sub> output from day 15 endometrium between 12h and 18h, and 18h and 24h of culture (Figure 3.6.j).

The PGF<sub>2α</sub> synthesizing capacity of day 15 guinea-pig endometrium after 6h of culture was significantly ( $P < 0.05$ ) higher than at 0h following treatment with 1000ng oestradiol/ml, and was also increased, but not significantly, following treatment with 10ng oestradiol/ml (Figure 3.6.k). After 12h, the PGF<sub>2α</sub> synthesizing capacity of oestradiol-treated, day 15 endometrium was significantly ( $P < 0.05$ ) higher than at 0h, although 1000ng oestradiol/ml treatment significantly ( $P < 0.05$ ) reduced PGF<sub>2α</sub> synthesizing capacity compared to the 12h control value. After 18h, the PGF<sub>2α</sub> synthesizing capacity of day 15 endometrium treated with oestradiol (10ng/ml) was significantly ( $P < 0.05$ ) higher than the 0h control value. Treatment with oestradiol (1000ng/ml) significantly ( $P < 0.05$ ) reduced PGF<sub>2α</sub> synthesizing capacity after 18h and 24h of culture compared to the respective control value at the same time. Treatment with 10ng oestradiol/ml prevented the increase in PGE<sub>2</sub> synthesizing capacity of day 15 endometrium after 6h and 24h of culture, but had no significant effect after 12h and 18h of culture (Figure 3.6.k). Oestradiol (1000ng/ml) treatment significantly ( $P < 0.05$ ) prevented the rise in PGE<sub>2</sub> synthesizing capacity of day 15 endometrium after 6h, 18h and 24h of culture, but not after 12h of culture, and significantly ( $P < 0.05$ ) reduced PGE<sub>2</sub> synthesizing capacity at every sample period compared to the respective control value at the same time (Figure 3.6.k). Oestradiol treatment (10ng/ml) had no inhibitory effect on the

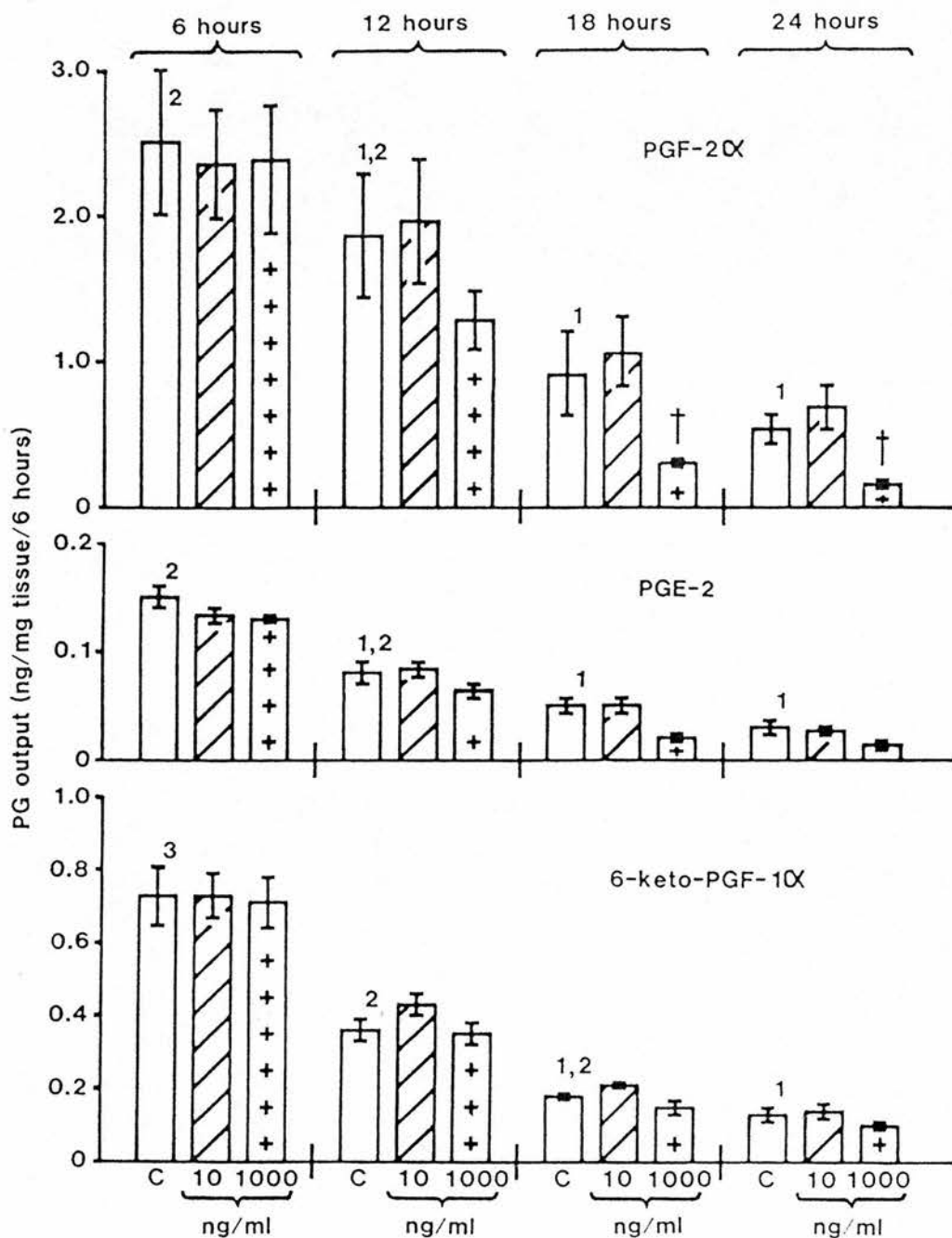


Figure 3.6.j

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  (ng/mg tissue/6h) from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h, not treated (controls (C); open columns), or treated with 10ng oestradiol/ml (hatched columns), or 1000ng oestradiol/ml (crossed columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG output at the same time for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

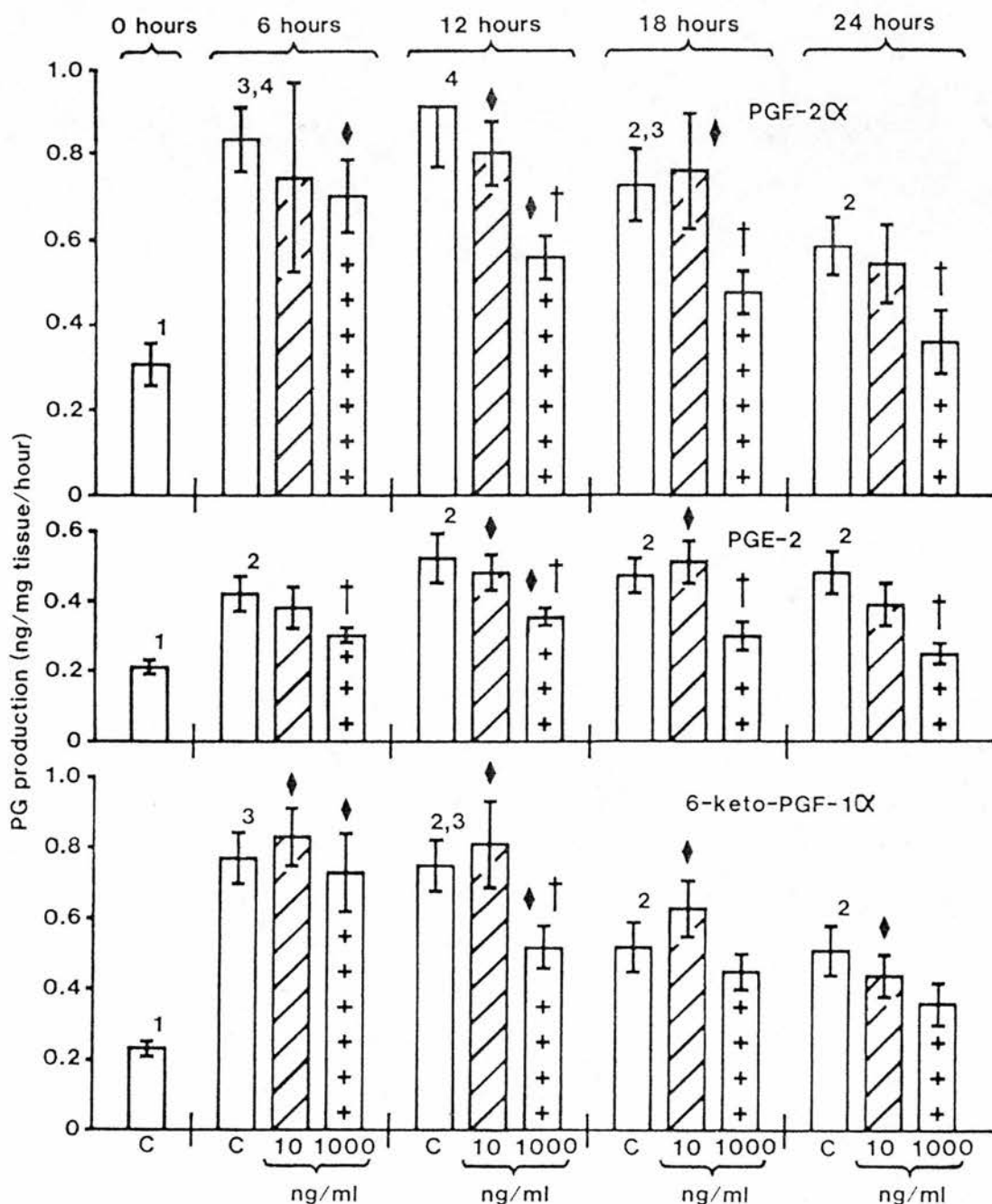


Figure 3.6.k

Mean ( $\pm$  s.e.m.;  $n = 6$ ) amounts of PGF $_2\alpha$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  (ng/mg tissue/h) synthesised by homogenates of day 15 guinea-pig endometrium at 0h and after 6h, 12h, 18h and 24h of culture, not treated (control (C); open columns), or treated with 10ng oestradiol/ml (hatched columns) or 1000ng oestradiol/ml (crossed columns) during the culture period.

† significantly ( $P < 0.05$ ) lower than the control (C) PG production at the same time for the same PG.

♦ significantly ( $P < 0.05$ ) higher than the 0h control (C) PG production for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

increase in 6-keto-PGF<sub>1α</sub> synthesizing capacity of day 15 endometrium observed during the 24h period of culture (Figure 3.6.k). However, oestradiol (1000ng/ml) treatment prevented this increase in 6-keto-PGF<sub>1α</sub> synthesizing capacity after 18h and 24h, but not after 6h of culture, and furthermore, this treatment significantly ( $P < 0.05$ ) reduced 6-keto-PGF<sub>1α</sub> synthesizing capacity after 12h of culture when compared to the control value at the same time (Figure 3.6.k).

Progesterone treatment: Progesterone (10 and 100ng/ml) treatment significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub> output from day 7 endometrium between 12h and 18h, and 18h and 24h of culture when compared to the corresponding control values at the same times (Figure 3.6.l). This inhibitory action of progesterone prevented the rise in PGF<sub>2α</sub> output observed between 12h and 24h in control values over the same time period. Progesterone (both concentrations) treatment had no significant effect on PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs when compared to the respective control values at the same times (Figure 3.6.l).

Treatment with both concentrations of progesterone (10 and 100ng/ml) reduced and delayed the increase in PGF<sub>2α</sub> synthesizing capacity of homogenates of day 7 endometrium during culture for 24h, when compared to control values at the same times (Figure 3.6.m). Unlike the control PGF<sub>2α</sub> synthesizing capacity value after 12h, 18h and 24h of culture, the PGF<sub>2α</sub> synthesizing capacity of day 7 endometrium treated with progesterone was not significantly higher than the 0h control value at any sample time. In fact, progesterone treatment significantly ( $P < 0.05$ ) lowered PGF<sub>2α</sub> synthesizing capacity (except for 100ng progesterone/ml treatment after 24h of culture) when compared to the respective control value at the same time. Progesterone treatment prevented the significant ( $P < 0.05$ ) rise in PGE<sub>2</sub> synthesizing capacity of day 7 endometrium during 24h of culture.



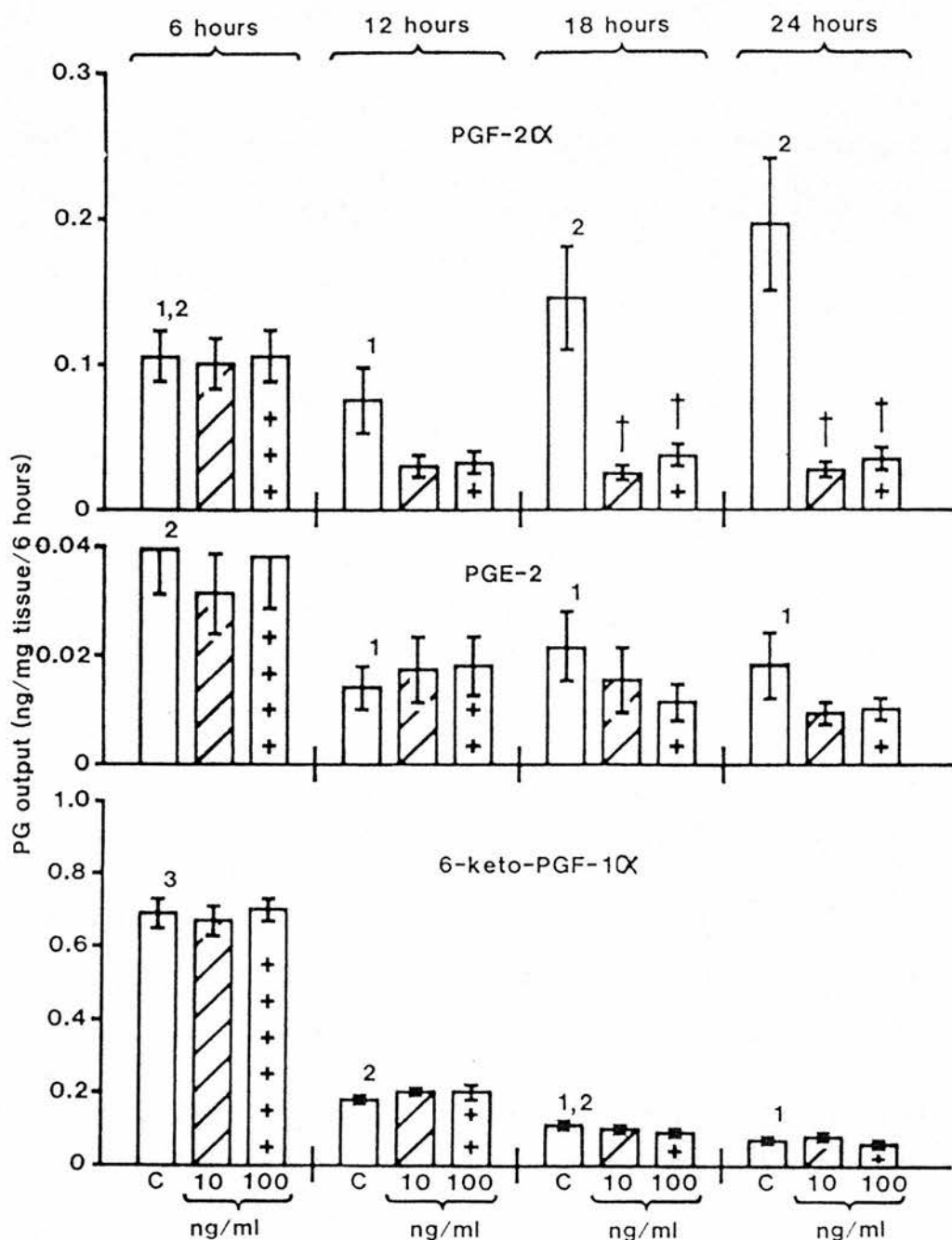


Figure 3.6.1

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  (ng/mg tissue/6h) from guinea-pig endometrium removed on day 7 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h, not treated (controls (C); open columns), or treated with 10ng progesterone/ml (hatched columns), or 100ng progesterone/ml (crossed columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG output at the same time for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.



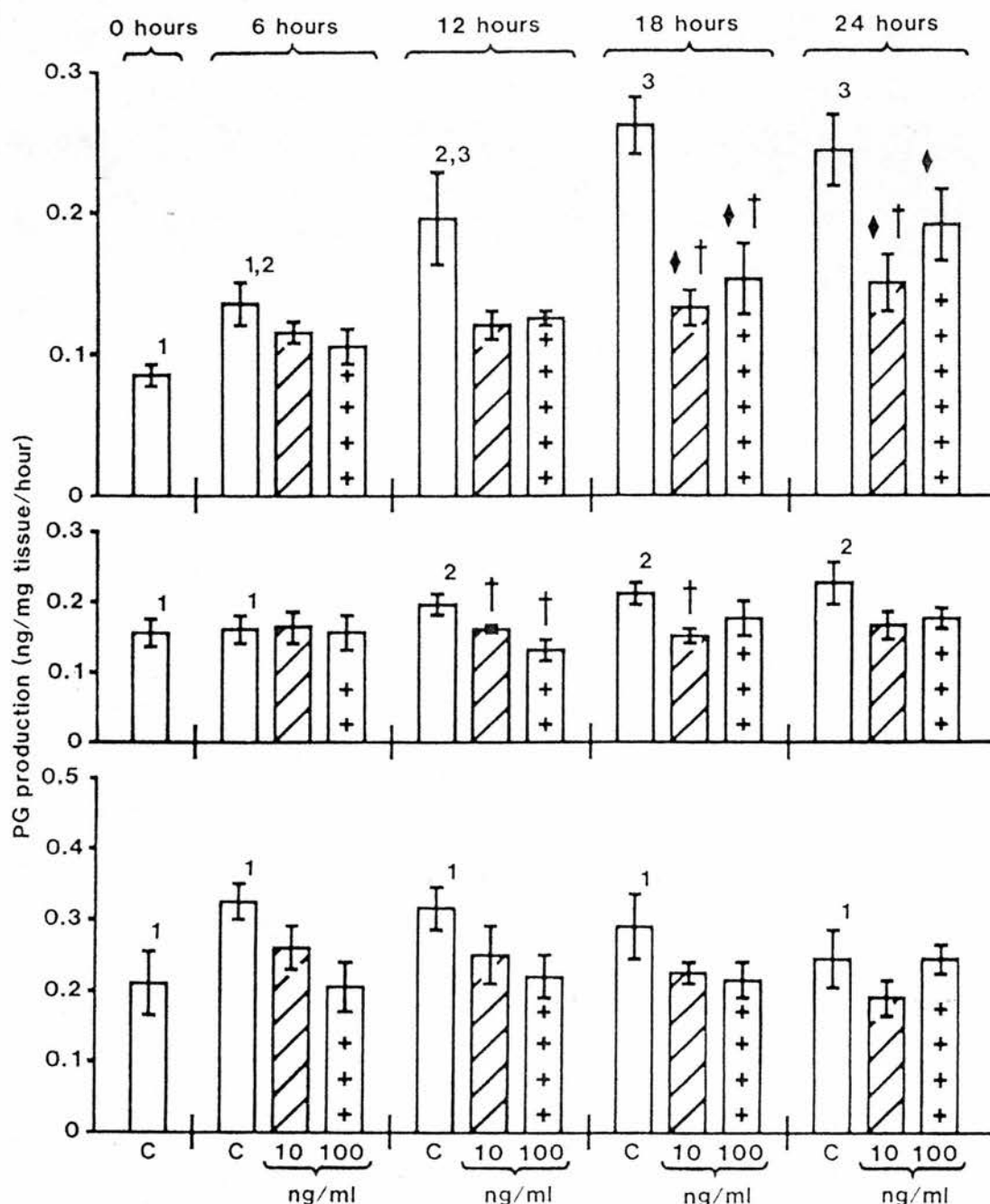


Figure 3.6.m

Mean ( $\pm$  s.e.m.;  $n = 6$ ) amounts of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α (ng/mg tissue/h) synthesised by homogenates of day 7 guinea-pig endometrium at 0h and after 6h, 12h, 18h and 24h of culture, not treated (control (C); open columns), or treated with 10ng progesterone/ml (hatched columns) or 100ng progesterone/ml (crossed columns) during the culture period.

† significantly ( $P < 0.05$ ) lower than the control (C) PG production at the same time for the same PG.

♦ significantly ( $P < 0.05$ ) higher than the 0h control (C) PG production for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

The PGE<sub>2</sub> synthesizing capacity of day 7 endometrium was significantly ( $P < 0.05$ ) lower than the corresponding control value at the same time, after 12h of culture when treated with progesterone (10 and 100ng/ml) and after 18h of culture when treated with 10ng progesterone/ml (Figure 3.6.m). The 6-keto-PGF<sub>2</sub> $\alpha$  synthesizing capacity of day 7 endometrium was not significantly changed by progesterone treatment throughout the 24h culture period (Figure 3.6.m).

The output of PGF<sub>2</sub> $\alpha$  from day 15 endometrium cultured for 24h was significantly ( $P < 0.05$ ) inhibited by progesterone (10 and 100ng/ml) treatment, between 12h and 18h, and 18h and 24h when compared to the control values during the same time period (Figure 3.6.n). The outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub> $\alpha$  were not altered by progesterone treatment, except PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) inhibited by 10ng progesterone/ml between 12h and 18h, and 6-keto-PGF<sub>1</sub> $\alpha$  output was significantly ( $P < 0.05$ ) inhibited by 100ng progesterone/ml between 18h and 24h of culture (Figure 3.6.n).

The rise in PGF<sub>2</sub> $\alpha$  synthesizing capacity during the 24h culture of day 15 endometrium demonstrated by the control values was prevented by progesterone treatment at both concentrations used (10 and 100ng/ml; Figure 3.6.o). During 24h of culture, the amounts of PGF<sub>2</sub> $\alpha$  produced by homogenates of day 15 endometrium following treatment with progesterone were not significantly higher than the 0h control value. After 6h, the PGF<sub>2</sub> $\alpha$  synthesizing capacity of day 15 endometrium treated with progesterone (10 and 100ng/ml) was neither significantly higher than the 0h control value nor significantly lower than the 6h control value, but was between both control values. However, after 12h, 18h and 24h of culture, the PGF<sub>2</sub> $\alpha$  synthesizing capacities of day 15 endometrium treated with both concentrations of

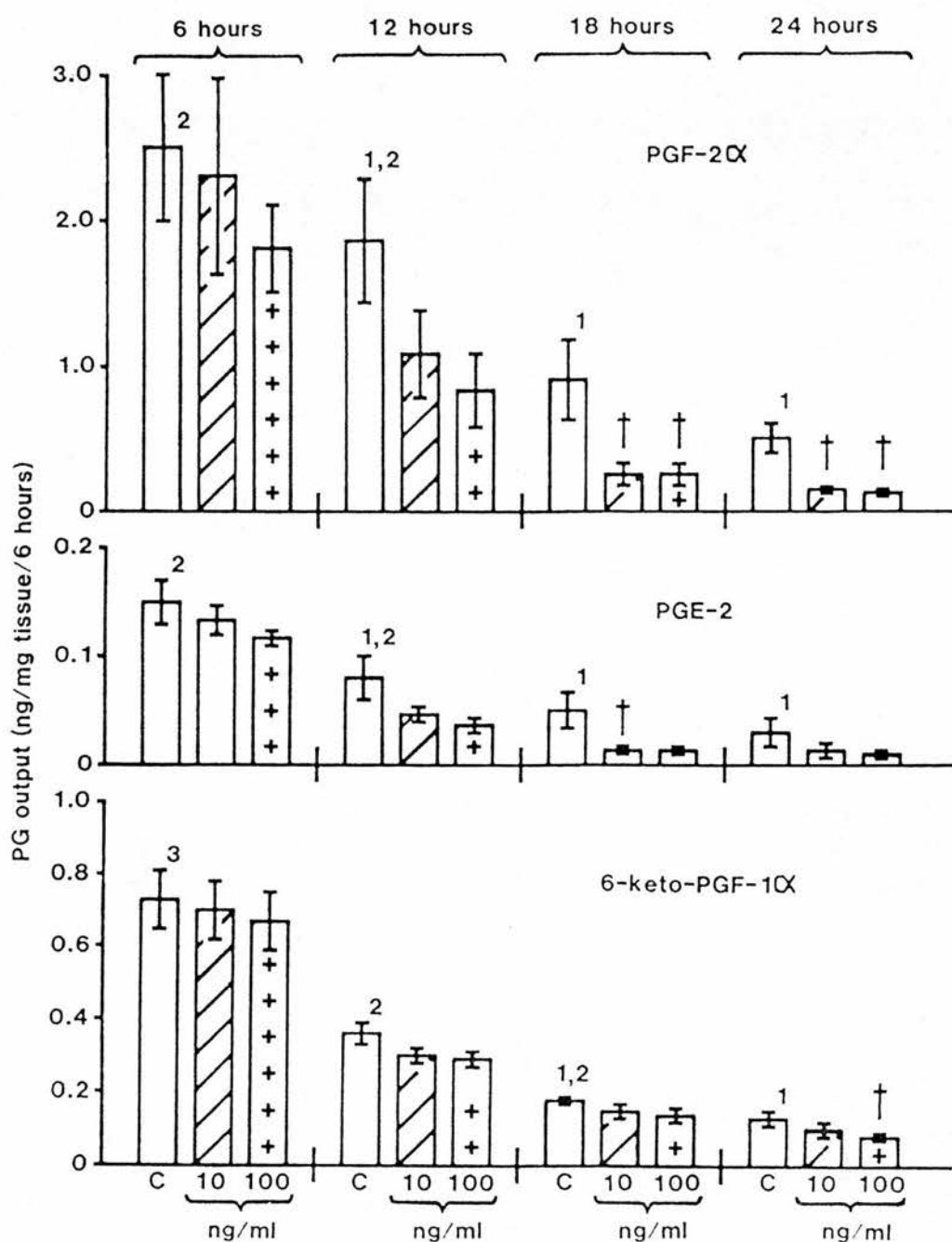


Figure 3.6.n

Mean ( $\pm$  s.e.m.;  $n = 6$ ) outputs of PGF<sub>2</sub>α, PGE<sub>2</sub> and 6-keto-PGF<sub>1</sub>α (ng/mg tissue/6h) from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured for up to 24h, changing the culture medium every 6h, not treated (controls (C); open columns), or treated with 10ng progesterone/ml (hatched columns), or 100ng progesterone/ml (crossed columns).

† significantly ( $P < 0.05$ ) lower than the control (C) PG output at the same time for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

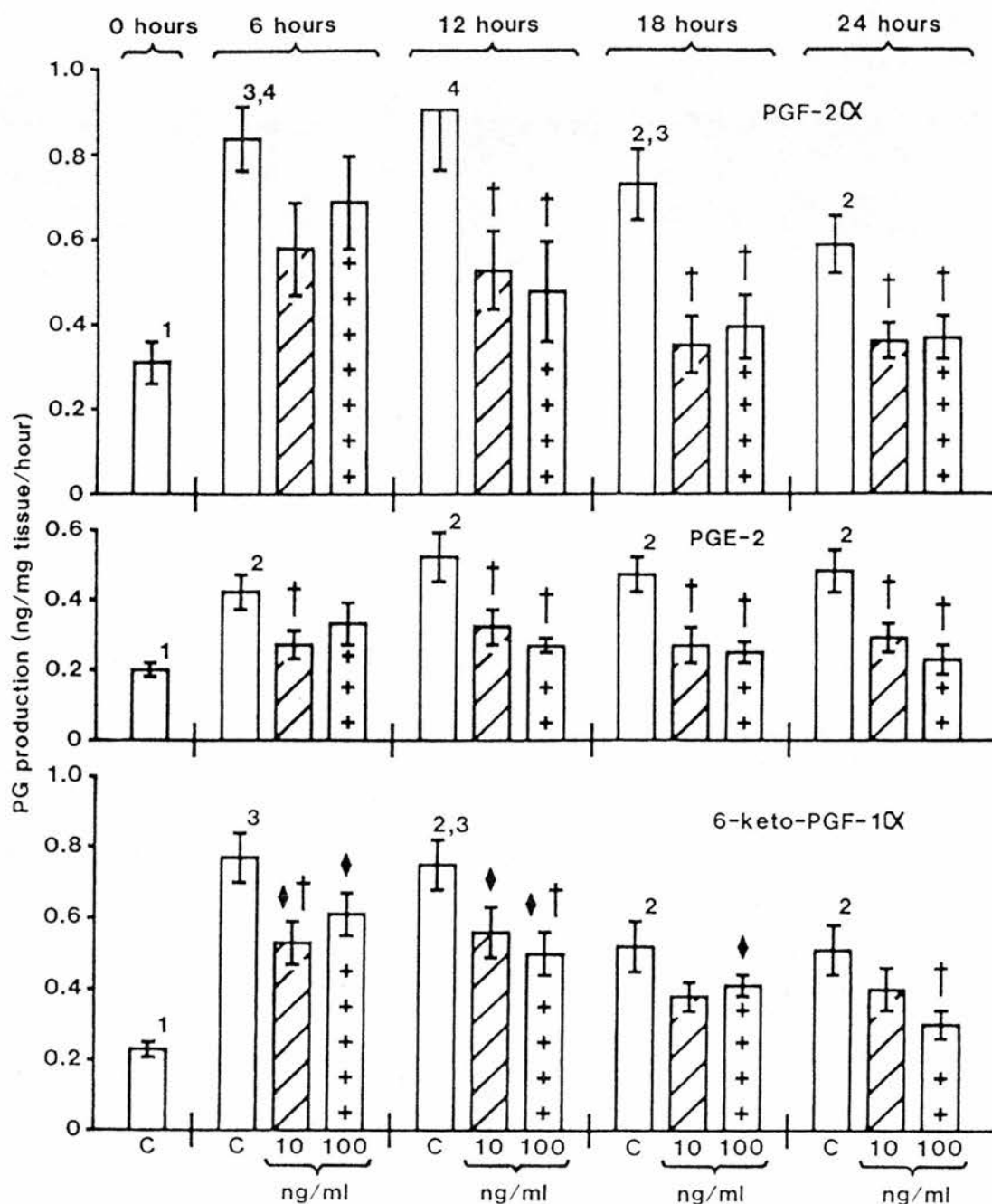


Figure 3.6.o

Mean ( $\pm$  s.e.m.;  $n = 6$ ) amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (ng/mg tissue/h) synthesised by homogenates of day 15 guinea-pig endometrium at 0h and after 6h, 12h, 18h and 24h of culture, not treated (control (C); open columns), or treated with 10ng progesterone/ml (hatched columns) or 100ng progesterone/ml (crossed columns) during the culture period.

† significantly ( $P < 0.05$ ) lower than the control (C) PG production at the same time for the same PG.

♦ significantly ( $P < 0.05$ ) higher than the 0h control (C) PG production for the same PG.

Control (C) columns with different superscripts for the same PG indicate the values are significantly ( $P < 0.05$ ) different.

progesterone were significantly ( $P < 0.05$ ) lower than their respective control value at the same times. Progesterone treatment (both concentrations used) also prevented the rise in  $\text{PGE}_2$  synthesizing capacity of day 15 endometrium that occurred during culture (Figure 3.6.o). The  $\text{PGE}_2$  synthesizing capacity of day 15 endometrium after 6h, 12h, 18h and 24h of culture was significantly ( $P < 0.05$ ) inhibited by progesterone treatment (10 and 100ng/ml) when compared to their respective control values at the same times, except after 6h of culture for the 100ng progesterone/ml treatment. The 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 15 endometrial homogenates was less affected by progesterone treatment than the synthesizing capacities of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  (Figure 3.6.o). After 6h and 12h of culture, the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 15 endometrium treated with both concentrations of progesterone was significantly ( $P < 0.05$ ) higher than the 0h control value. However, the treatment of day 15 endometrium with 10ng progesterone/ml for 6h, and with 100ng progesterone/ml for 12h significantly ( $P < 0.05$ ) reduced the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity compared to the corresponding control values at the same time. After 18h and 24h of culture, the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of progesterone-treated, day 15 endometrium was not significantly higher (unlike control values at these times) than the 0h control value, except for the value for day 15 endometrium treated with 100ng progesterone/ml for 18h of culture which was significantly ( $P < 0.05$ ) raised. Also, the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 15 endometrium treated with 100ng progesterone/ml for 24h of culture was significantly ( $P < 0.05$ ) lower than the corresponding 24h control value (Figure 3.6.o).

#### Experiment 4

The incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted

proteins synthesised by day 7 and day 15 guinea-pig endometrium in culture for 24h, and not treated (control) or treated with oestradiol and progesterone, is shown in Figure 3.6.p. There was no significant difference between the amounts of [ $^3\text{H}$ ]-leucine incorporated into total cellular proteins synthesised by day 7 and day 15 endometrium cultured for 24h. The incorporation of [ $^3\text{H}$ ]-leucine into secreted proteins however, was significantly ( $P < 0.05$ ) higher by day 15 than by day 7 endometrium. These untreated (control) results are comparable with the previously presented profiles of incorporation of [ $^3\text{H}$ ]-leucine into cellular and secreted proteins after 24h of culture (see section 3.4 and Figure 3.4.a).

Oestradiol and progesterone treatment had no significant effect on the incorporation of [ $^3\text{H}$ ]-leucine into cellular proteins by day 7 and day 15 endometrium in culture for 24h. However, 1000ng oestradiol/ml, but not 10ng oestradiol/ml, significantly ( $P < 0.05$ ) inhibited the incorporation of [ $^3\text{H}$ ]-leucine into secreted proteins by both day 7 and day 15 cultured endometrium. Furthermore, progesterone (10 and 100ng/ml) treatment of day 15 endometrium, but not of day 7 endometrium, significantly ( $P < 0.05$ ) reduced the incorporation of [ $^3\text{H}$ ]-leucine into secreted proteins during 24h in culture (Figure 3.6.p).

### CONCLUSIONS

The untreated, control, basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium cultured for three days and for 24h were comparable to the control PG outputs during these periods of culture, presented earlier in Section 3.1. The output of  $\text{PGF}_{2\alpha}$  was higher from day 15 than from day 7 endometrium.  $\text{PGF}_{2\alpha}$  output declined from both day 7 and day 15 endometrium when

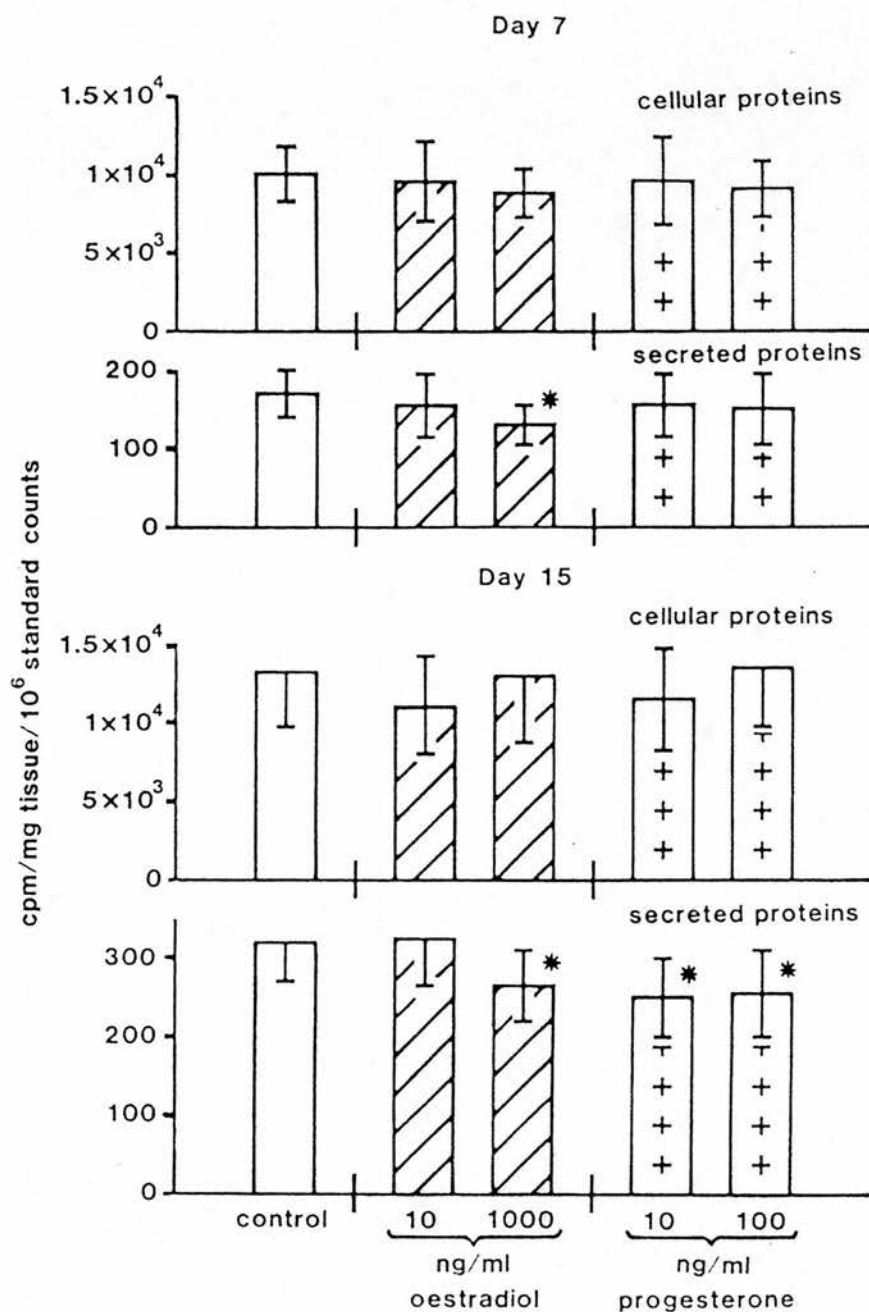


Figure 3.6.p

Mean ( $\pm$  s.e.m.;  $n = 5$ ) amounts of [ $^3$ H]-leucine (measured as cpm/mg tissue/ $10^6$  standard counts) incorporated into cellular and secreted proteins synthesised by day 7 and day 15 guinea-pig endometrium in culture for 24h, not treated (controls; open columns) or treated with oestradiol (10 and 1000ng/ml; hatched columns) or progesterone (10 and 100ng/ml; crossed columns).

\* significantly ( $P < 0.05$ ) lower than [ $^3$ H]-leucine incorporation into control secreted proteins by endometrium removed on the same day of the cycle (by the paired 't' test).

cultured for three days, and  $\text{PGF}_{2\alpha}$  output declined from day 15 but not from day 7 endometrium when cultured for 24h. This decline in output during either of the culture periods was not overcome by oestradiol treatment. Day 7 endometrium exhibited an increase in  $\text{PGF}_{2\alpha}$  output during the final 12h of the 24h culture period. Oestradiol treatment had no stimulatory effect on this output either. Furthermore, treatment of cultured guinea-pig endometrium with oxytocin had no stimulatory action on  $\text{PGF}_{2\alpha}$  output.

Indeed, oestradiol, at the highest concentration (1000ng/ml) used was found to inhibit  $\text{PGF}_{2\alpha}$  output. The highest concentration of oestradiol (1000ng/ml) also inhibited  $\text{PGE}_2$ , but not 6-keto- $\text{PGF}_{1\alpha}$  output from both day 7 and day 15 endometrium in culture. This high dose of oestradiol also had an inhibitory effect on the  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  synthesizing capacities of day 7 and day 15 guinea-pig endometrium in culture and had a slight inhibitory action on the 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity of day 15 endometrium.

Progesterone treatment inhibited  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ , but not 6-keto- $\text{PGF}_{1\alpha}$  basal outputs from both day 7 and day 15 guinea-pig endometrium in culture, during both three days and 24h of culture. Progesterone also had a marked inhibitory action on  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  synthesizing capacities of both day 7 and day 15 endometrium, but very little effect on the day 15, and no effect on the day 7 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity.

Hydrocortisone, which is the main glucocorticoid hormone secreted in the guinea-pig (Fajer and Vogt, 1963), had no effect on any PG output from day 15 endometrium on any day of culture. Furthermore, progesterone exhibited an inhibitory action on PG production, whereas hydrocortisone had no effect. Therefore, it is unlikely that the inhibitory action of progesterone occurs via a



glucocorticoid-like effect.

Oestradiol, at the highest concentration used (1000ng/ml) had an inhibitory action on the synthesis of secreted proteins, but not on cellular proteins, synthesised by both day 7 and day 15 guinea-pig endometrium cultured for 24h. Progesterone treatment (10 and 100ng/ml) had an inhibitory effect on the synthesis of secreted, but not cellular proteins, by day 15 but not by day 7 endometrium in culture for 24h.

## DISCUSSION

In Experiments 1, 2 and 3 the output of  $\text{PGF}_{2\alpha}$  during the first 24h of culture was approximately 10-fold greater from day 15 endometrium than from day 7 endometrium. This increase is consistent with the in vivo stimulatory action of oestradiol, released in increasing amounts from the ovary after day 11 (Joshi, Watson and Labhsetwar, 1973), acting on a progesterone-primed uterus causing this increase in  $\text{PGF}_{2\alpha}$  output between day 7 and day 15 of the oestrous cycle.

Oestradiol and oxytocin did not stimulate  $\text{PGF}_{2\alpha}$  output from the guinea-pig endometrium in culture. These findings are contrary to a previous study by Leaver and Seawright (1982), who reported that both hormones stimulated  $\text{PGF}_{2\alpha}$  output from cultured guinea-pig endometrium. However, this lack of effect on PG production by oxytocin is consistent with work by Poyser and Brydon (1983) who showed there was no effect on PG outputs from the guinea-pig uterus during a 30 min superfusion with oxytocin. Poyser and Brydon (1983) and Poyser (1987b) demonstrated that treatment with oestradiol of both day 7 and day 15 guinea-pig uterine horns superfused in vitro also had no effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ , 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{TXB}_2$ .

Illingworth and Perry (1973) reported that large, non-physiological doses of oestradiol administered to guinea-pigs on day 9 of the cycle prevented luteolysis due, at least in part, to an anti-luteolytic effect. In the present study, oestradiol at the highest concentration used (1000ng/ml) inhibited  $\text{PGF}_{2\alpha}$  output and also  $\text{PGE}_2$  output from the cultured guinea-pig endometrium, and this inhibitory action on the synthesis of the uterine luteolytic hormone in vitro may explain the anti-luteolytic effect of oestradiol observed in vivo.

There were several discrepancies in the results from Experiment 2 when comparing PG outputs with the corresponding PG synthesizing capacity which suggests that the PG synthesizing capacity is not the factor controlling basal PG output. The profiles of control basal  $\text{PGF}_{2\alpha}$  output and of  $\text{PGF}_{2\alpha}$  synthesizing capacity of day 7 endometrium in culture for 24h exhibited some differences. The basal output of  $\text{PGF}_{2\alpha}$  during the 24h period declined between 0-6h and 6h-12h, then increased to the end of the culture period, whereas  $\text{PGF}_{2\alpha}$  synthesizing capacity increased from 0h and plateaued at a level that was significantly higher than the 0h level. Furthermore, the profiles of the basal outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from control day 7 endometrium declined during the 24h in culture which differed from their respective synthesizing capacity profiles: the synthesizing capacity of  $\text{PGE}_2$  increased during the culture period from the 0h value, and 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacity was maintained during the culture period at a constant level. The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium all declined throughout the 24h culture period. However, the synthesizing capacity of all three PGs increased between 0h and 6h, and then the  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$ , but not the  $\text{PGE}_2$  synthesizing capacities declined only slightly, so showing very different profiles of PG synthesizing

capacity for cultured endometrium compared to the respective profiles of PG output. Treatment of day 7 endometrium with oestradiol (1000ng/ml) prevented the increase in  $\text{PGF}_{2\alpha}$  output between 12h and 18h, and 18h and 24h of culture, whereas  $\text{PGF}_{2\alpha}$  synthesizing capacity after 18h and 24h was significantly higher than the 0h control value (although the  $\text{PGF}_{2\alpha}$  synthesizing capacity after 18h was significantly lower than the corresponding control value at the same time). The synthesizing capacities of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from day 7 endometrium treated with 10ng oestradiol/ml were significantly higher after 18h of culture than their respective controls at the same time, but the outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  between 12h and 18h after oestradiol (10ng/ml) treatment were not changed from their control values. Oestradiol treatment (1000ng/ml) significantly decreased  $\text{PGE}_2$  synthesizing capacity from day 15 endometrium at every sample time, but the output of  $\text{PGE}_2$  was unaffected throughout the 24h culture period. Treatment of day 7 endometrium with progesterone (10 and 100ng/ml) prevented the increase in basal  $\text{PGF}_{2\alpha}$  output, but did not prevent the increase in  $\text{PGF}_{2\alpha}$  synthesizing capacity (although the synthesizing capacity values were still lower than control values). Progesterone treatment (10 and 100ng/ml) significantly inhibited both  $\text{PGF}_{2\alpha}$  output and synthesizing capacity from cultured day 15 endometrium, but the progesterone inhibitory action on  $\text{PGF}_{2\alpha}$  synthesizing capacity occurred after 12h of culture, whereas the inhibition of  $\text{PGF}_{2\alpha}$  output was observed in the subsequent sample periods between 12h and 24h of culture. This evidence (also see section 3.2 and 3.5 for further similar evidence) has shown that the synthesizing capacity (and hence endometrial PGH synthase concentrations) is not the controlling step in PG synthesis by the guinea-pig endometrium.

Treatment with glucocorticoids in some tissues, including the human endometrium in tissue culture (Gurpide, Markiewicz, Schatz and Hirata, 1986), increase the production of proteins called lipocortins, that inhibit  $PLA_2$  activity. As hydrocortisone treatment had no effect on PG production from the guinea-pig endometrium in culture, this suggests lipocortins are not involved in the control of  $PLA_2$  activity, which is the rate-limiting step in the control of PG synthesis (Vogt, 1978). As progesterone exhibits an inhibitory effect on PG production, the progesterone action is not likely to be due to a glucocorticoid-like action. This finding is in agreement with Schatz, Markiewicz and Gurpide (1986) who demonstrated that both the glucocorticoid (dexamethasone) and progesterone inhibit  $PGF_{2\alpha}$  output from cultured human endometrium. However, dexamethasone increased the amount of lipocortin released by the endometrium into the culture medium, whereas progesterone decreased lipocortin production. Therefore, progesterone and dexamethasone do not inhibit the output of  $PGF_{2\alpha}$  from human endometrium in culture by the same mechanism.

To summarise the findings from this results section, oestradiol and oxytocin did not possess a stimulatory action on  $PGF_{2\alpha}$  output from cultured guinea-pig endometrium <sup>contrary to</sup> a previous report. In fact oestradiol at the highest dose administered inhibited both  $PGF_{2\alpha}$  output and synthesizing capacity. Hydrocortisone had no effect on PG output, providing further evidence that lipocortin is not involved in the control of  $PLA_2$  activity and hence PG synthesis. Progesterone inhibited  $PGF_{2\alpha}$  production, but this effect appears not to be a glucocorticoid-like action. Discrepancies between the PG output and the corresponding PG synthesizing capacity provide further evidence that tissue PGH synthase levels are not the controlling step in PG

synthesis by guinea-pig endometrium. Both progesterone and oestradiol inhibited the synthesis of secreted proteins by the day 15 guinea-pig endometrium when  $\text{PGF}_{2\alpha}$  output was high. Therefore a protein may be involved in the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis towards the end of the oestrous cycle.

## SECTION 4

### GENERAL DISCUSSION

The basal output of  $\text{PGF}_{2\alpha}$  was higher from the Day 15 guinea-pig uterus than from the day 7 guinea-pig uterus, as demonstrated by the superfusion experiments (Section 3.5). The site of production of the uterine luteolytic hormone is the endometrium (Poyser, 1983a) and, in culture, endometrium removed from day 15 guinea-pigs produced more  $\text{PGF}_{2\alpha}$  than endometrium removed from day 7 animals. These findings agree with previous studies which examined  $\text{PGF}_{2\alpha}$  output from cultured guinea-pig endometrium (Leaver and Seawright, 1982) and from guinea-pig uterine horns superfused in vitro (Poyser, 1983b; Poyser and Brydon, 1983), and reflect the higher  $\text{PGF}_{2\alpha}$  concentrations observed in the guinea-pig uterine venous plasma that occur towards the end of the oestrous cycle (Blatchley, Donovan, Horton and Poyser, 1972).

The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 guinea-pig uteri superfused in vitro and from day 15 cultured endometrium were much lower than the output of  $\text{PGF}_{2\alpha}$ , as previously reported (Poyser and Brydon, 1983). The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 superfused guinea-pig uteri and from day 7 cultured endometrium were similar to their respective outputs from day 15 tissue, and were also similar to the output of  $\text{PGF}_{2\alpha}$  from the day 7 superfused uterus and cultured endometrium, and these results agree with previous reports (Poyser, 1983b; Poyser and Brydon, 1983).

The amounts of PGs measured as "output" from uterine tissues is not only dependent upon PG synthesis but is also dependent upon the catabolism of PGs. The amounts of PGs measured in incubates of homogenates of uterine tissues (as a measure of tissue PGH synthase levels; synthesizing capacity) is also dependent on the catabolism of PGs in the incubates. The initial reaction in PG catabolism is

catalysed by  $\text{NAD}^+$ -dependent 15-hydroxyprostaglandin dehydrogenase. In the pig lung, no measurable metabolism to the 15-keto-PG product occurred when  $\text{NAD}^+$  was absent (Anggard and Samuelsson, 1966). Subsequent catabolism by  $\Delta^{13}$ -PG reductase, which requires NADPH as a co-factor, produces the 13,14-dihydro-15-keto-PG product.

The antisera used to measure  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  by radioimmunoassay in these studies have very low cross-reactivities with the 15-keto-PG and 13,14-dihydro-15-keto-PG metabolites (see Tables 2.3.2.i, 2.3.3.i and 2.3.4.i for the cross-reactivities of PG antibodies raised to  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ , respectively). Clearly, the radioimmunoassay antisera used do not cross-react with the PG metabolites, but uterine tissue must be examined to determine whether measured PGs reflect total PG production or only a fraction of the total PG production because of PG catabolism.

Injected PGs are rapidly metabolised during a single passage through the lungs (Ferreira and Vane, 1967), and metabolising enzymes have also been identified in the testis (Nakano and Prancan, 1971), liver (Dawson, Ramwell and Shaw, 1968), kidney (Nissen and Andersen, 1968) and placenta (Jarabak, 1974). In human uterine homogenates, Nakano, Montague and Darrow (1971) found that the metabolism of  $\text{PGE}_1$  was low. Subsequently however, Casey, Hemsell, MacDonald and Johnston (1980) reported that the specific activity of  $\text{NAD}^+$ -dependent 15-hydroxyprostaglandin dehydrogenase, measured using  $\text{PGE}_2$  as substrate, was 55-fold greater in endometrium during the secretory phase when compared to the menstrual and premenstrual stages, and that the activity during the secretory phase was 3.5-fold greater than during the proliferative phase. Prostaglandin dehydrogenase activity was primarily found in the cytosolic fractions of glandular but not stromal epithelia (Casey, Hemsell, MacDonald and Johnston, 1980).

However, without the addition of  $\text{NAD}^+$ , or with the addition of the reduced form of the co-factor (NADH), homogenates of human uterine tissue did not exhibit any measurable catabolism (Abel and Kelly, 1983).

In the guinea-pig, Maule Walker and Poyser (1978) demonstrated that exogenous  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  were almost completely metabolised when added to homogenates of day 15 guinea-pig lung when incubated in Tyrode's solution containing  $\text{NAD}^+$ . This finding is in agreement with in vivo observations by Piper, Vane and Wyllie (1970) who demonstrated the lungs appeared to be the principle site of PG metabolism in the guinea-pig. Metabolism after the addition of exogenous  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  to day 15 guinea-pig uterine homogenates was relatively low (up to 14% metabolism) with  $\text{NAD}^+$  present in the incubates (Maule Walker and Poyser, 1978). Without  $\text{NAD}^+$  present, no detectable metabolism (< 5%) of  $\text{PGF}_{2\alpha}$  occurred in guinea-pig uterine homogenates (Poyser, 1979). These findings show that there is no significant metabolism of PGs by guinea-pig uterine tissue, and PG outputs and PG synthesizing capacities reflect the total PG release from and PG synthesis by the uterus.

Where is the specific site of  $\text{PGF}_{2\alpha}$  synthesis? In the human uterus, both glandular and stromal cells synthesise PGs (Schatz and Gurpide, 1983), but there is some disagreement as to which cell type is the main source. In non-pregnant endometrium maintained in organ culture for 10-21 days, Gal, Casey, Johnston and MacDonald (1982) reported that stromal cells released more PGs than glandular cells. However, in this study over such a long time period, it must be questioned whether these stromal and glandular cells reflect their true state in vivo. However, Lumsden, Brown and Baird (1983) found that homogenates of glands from either secretory or proliferative



endometrium converted more [ $^{14}\text{C}$ ]-arachidonic acid to  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  than stromal cells, and that the glandular cells released more PGs than the stromal cells.

Oestradiol ( $10^{-8}\text{M}$ ) stimulated PGF production 4-fold during a 24h incubation of human glandular tissue, whereas stromal cell PGF production was either unaffected or inhibited (Skinner, Liggins, Wilson and Neale, 1984). Schatz, Markiewicz and Gurside (1986) demonstrated that the basal output of  $\text{PGF}_{2\alpha}$  was similar from both endometrial epithelial (glandular) and stromal cells, but oestradiol only stimulated  $\text{PGF}_{2\alpha}$  output from epithelial cells. The response of epithelial cells to oestradiol of increased  $\text{PGF}_{2\alpha}$  synthesis was further stimulated by A23187 administration, but A23187 had no effect on  $\text{PGF}_{2\alpha}$  output from stromal cells (Schatz, Markiewicz and Gurside, 1987). It was concluded that both cell types from the human endometrium may contribute significantly to basal  $\text{PGF}_{2\alpha}$  output, but that glandular epithelial cells, not stromal cells, are the primary targets for the stimulatory effect of oestradiol on  $\text{PGF}_{2\alpha}$  output by secretory endometrium (Schatz, Markiewicz and Gurside, 1987).

The immunocytochemical localisation of PGH synthase has suggested that this enzyme is limited to the epithelial component of secretory endometrium (Rees, Parry, Anderson and Turnbull, 1982), although some PGH synthase must exist in stromal cells for PG production. Within the cell, Rollins and Smith (1980), in an electron microscope study of mouse Swiss 3T3 cells, determined that monoclonal antibodies raised against PGH synthase bound to the endoplasmic reticulum and membranes adjoining the outer nuclear membrane, but not to plasma or mitochondrial membranes. Subsequently, DeWitt, Rollins, Day, Gauger and Smith (1981) localised PGH synthase to the cytoplasmic surface of the endoplasmic reticulum of sheep vesicular gland microsomes. In

platelets, Carey, Menashi and Crawford (1982) also localised the site of PGH synthase to the endoplasmic reticulum. Also in platelets, the endoplasmic reticulum has been determined as the site of PLA<sub>2</sub> in the cell (Lagarde, Menashi and Crawford, 1981). The organisation and relationships of PLA<sub>2</sub>, PGH synthase and also the specific PG synthesizing enzymes within the endoplasmic reticulum is not known. PGF<sub>2α</sub> synthesis by the guinea-pig endometrium is specifically stimulated towards the end of the cycle, and there is also a selective stimulation of PGF<sub>2α</sub> synthesizing capacity at the end of the cycle, particularly at oestrus when progesterone concentrations are lowest (Poyser, 1983a,b). This may suggest that discrete sites for the release of arachidonic acid by PLA<sub>2</sub>, for PGH synthase and for the PGF<sub>2α</sub> synthesizing apparatus are selectively organised to be in very close proximity towards the end of the cycle in order to allow the selective synthesis of PGF<sub>2α</sub>. Alternatively, if the enzymes for PG synthesis are not linked in this way for the synthesis of only one PG at discrete sites, as more arachidonic acid is released by PLA<sub>2</sub> after day 11 of the oestrous cycle, this extra precursor must be channelled by some mechanism specifically to the PGF<sub>2α</sub> synthesizing enzymes.

The next part of the general discussion examines the mechanisms by which PGF<sub>2α</sub>, and also PGE<sub>2</sub> and PGI<sub>2</sub>, synthesis and release is controlled in the guinea-pig uterus, and how uterine PGF<sub>2α</sub> production is stimulated towards the end of the oestrous cycle.

Extracellular calcium has been shown in the studies presented in this thesis to be required for PGF<sub>2α</sub> synthesis by the cultured guinea-pig endometrium. However, endometrial PGF<sub>2α</sub> synthesis may be dependent upon the release of Ca<sup>2+</sup> from intracellular stores, and the extracellular calcium may be required to replenish these stores rather than extracellular calcium acting directly to stimulate PGF<sub>2α</sub>

synthesis. Indeed, Poyser (1984a) showed that the basal output of  $\text{PGF}_{2\alpha}$  from day 15 guinea-pig uterus was not altered by superfusing in vitro with  $\text{Ca}^{2+}$ -free Krebs' solution for 150 min. It appears from these studies by Poyser (1984a) and the results presented here that the inhibitory effect of the removal of extracellular calcium on endometrial  $\text{PGF}_{2\alpha}$  synthesis occurs after several hours. This may be anticipated with the  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus being stimulated over several days in vivo towards the end of the oestrous cycle. Olson, Opavsky and Challis (1983) have reported that calcium, normally of extracellular source, is required for  $\text{PGE}_2$  production by dispersed human amnion cells, and this effect occurred during a 3h incubation. Intracellular  $\text{Ca}^{2+}$  may recycle to maintain high  $\text{PGF}_{2\alpha}$  output for short periods as demonstrated when using  $\text{Ca}^{2+}$ -free Krebs' superfusing solution which had no effect on uterine  $\text{PGF}_{2\alpha}$  output during a 2.5h superfusion (Poyser, 1984a). Alternatively, the stimulatory effect of calcium on  $\text{PGF}_{2\alpha}$  synthesis may be long-lasting and slow in onset, and this is in agreement with Poyser (1983b) who demonstrated that from the guinea-pig uterus superfused in vitro, basal  $\text{PGF}_{2\alpha}$  output was not inhibited by treatment for 40 min with TMB-8 (an intracellular  $\text{Ca}^{2+}$  antagonist). However, the stimulation of  $\text{PGF}_{2\alpha}$  output from the superfused guinea-pig uterus in vitro by calcium (by using the  $\text{Ca}^{2+}$  ionophore A23187) was eliminated using  $\text{Ca}^{2+}$ -free Krebs' solution (Poyser, 1984a) and was reduced by TMB-8 treatment. These findings demonstrate that  $\text{Ca}^{2+}$  is required for the further stimulation of  $\text{PGF}_{2\alpha}$  output above basal levels and that the response to this  $\text{Ca}^{2+}$  is fast (i.e. within minutes) in onset.

The removal of extracellular calcium from cultured day 15 endometrium had less of an inhibitory effect on basal  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs than on basal  $\text{PGF}_{2\alpha}$  output. This may be due to the

outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> being much lower than PGF<sub>2α</sub> output, and thus possibly having different controlling systems regulating their synthesis compared to the Ca<sup>2+</sup>-dependent PGF<sub>2α</sub> regulatory mechanism in the guinea-pig endometrium.

The high basal output of PGF<sub>2α</sub> from cultured day 15 guinea-pig endometrium was inhibited by the calmodulin inhibitors W-7 and, to a lesser extent, TFP. These results suggest that calmodulin is involved in the stimulation of PLA<sub>2</sub> activity and hence PGF<sub>2α</sub> synthesis. These findings are consistent with the inhibitory effects of TFP and W-7 on the A23187-stimulated increases in PGF<sub>2α</sub> output from the guinea-pig uterus superfused in vitro (Poyser, 1985a,b). Similarly, PGE<sub>2</sub> production by dispersed human amnion cells, removed by caesarian section prior to the onset of spontaneous labour, also show a similar requirement for calcium and calmodulin (Olson, Challis, Opavsky, Smeija, Kramar and Skinner, 1985; Olson, Kramar and Smeija, 1987).

How is the intracellular calcium concentration controlled ? Michell (1975) noted that accelerated phosphatidylinositol (PI) turnover was linked with cellular processes that are associated with Ca<sup>2+</sup> mobilisation. It was proposed that PI turnover might in some way trigger Ca<sup>2+</sup> mobilisation. Michell, Kirk, Jones, Downes and Creba (1981) suggested that the increased rate of turnover of phosphoinositides was a response of cells that reflected the transduction of signals across the cell membrane. Subsequently, the hydrolysis of phosphatidyl 4,5-bisphosphate by a phosphodiesterase to inositol 1,4,5-trisphosphate has been shown to be an important secondary messenger system in many cells which transduce the signal from an appropriate cell surface receptor, when an agonist binds to it, to release calcium from internal stores (Berridge, 1984; Berridge

and Irvine, 1984; Berridge, 1987)). This system is adapted to control short-term cellular responses. However, Ning and Poyser (1984) have demonstrated that there is no increase in turnover of phosphatidylinositol and phosphatidyl 4,5-bisphosphate in the guinea-pig endometrium on day 15 compared to day 7 of the oestrous cycle. Therefore, this signal mechanism is not involved in the control of intracellular calcium that controls PLA<sub>2</sub> activity and hence PG synthesis by the guinea-pig endometrium. This is not unexpected as the stimulus for PG synthesis towards the end of the cycle is oestradiol acting on a progesterone-primed uterus, and steroids usually exert their effect via an intracellular action by combining with a nuclear receptor and stimulating protein synthesis rather than by binding to an extracellular membrane receptor (see General Introduction).

The control of cAMP has also be postulated as a possible mechanism by which PG synthesis is controlled in the uterus. McCracken, Schramm and Okulicz (1984) developed a hypothesis to explain events leading to luteolysis in sheep. They proposed that oestradiol induces synthesis of oxytocin receptors which are inserted into the membranes of endometrial cells, and that oxytocin binding, by causing the stimulation of the adenylate cyclase system to generate cyclic adenosine monophosphate (cAMP), stimulates of PLA<sub>2</sub> activity and the release of arachidonic acid and, hence, increases PGF<sub>2</sub> $\alpha$  production. The action of many hormones are mediated within the target cell by activation of adenylate cyclase and cAMP production (Birnbaumer, 1977). The effect of forskolin, which stimulates adenylate cyclase, was examined on PG output from and cAMP production by the guinea-pig uterus (Poyser, 1987a). Forskolin stimulated cAMP production by the endometrium and myometrium, but had no effect on

uterine PG output. Therefore, cAMP is not involved in the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis and release. Also, as oxytocin treatment has no effect on PG output from cultured guinea-pig endometrium, as determined in these studies, and has no effect on PG output from the guinea-pig uterus superfused in vitro (Poyser and Brydon, 1983), the mechanism controlling  $\text{PGF}_{2\alpha}$  output from the sheep uterus involving oxytocin, as suggested by McCracken, Schramm and Okulicz (1984), does not control  $\text{PGF}_{2\alpha}$  synthesis by the guinea-pig uterus.

Fragments of human secretory endometrium in organ culture are stimulated by oestradiol to produce  $\text{PGF}_{2\alpha}$  (Abel and Baird, 1980; Tsang and Ooi, 1982; Schatz, Markiewicz and Gurside, 1984; Markiewicz, Schatz, Barg and Gurside, 1984; Schatz, Markiewicz, Barg and Gurside, 1985). Oestradiol also stimulates  $\text{PGF}_{2\alpha}$  production by human epithelial cells in monolayer culture, derived from glands of proliferative and secretory endometrium (Schatz and Gurside, 1983; Schatz, Markiewicz and Gurside, 1984; Schatz, Markiewicz, Barg and Gurside, 1985).

In the studies presented in this thesis, oestradiol had no stimulatory effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$  from the day 7 or day 15 guinea-pig endometrium in culture. These findings are contrary to a report by Leaver and Seawright (1982) who showed that oestradiol had a stimulatory effect on  $\text{PGF}_{2\alpha}$  output from both day 7 and day 15 cultured guinea-pig endometrium. The findings presented here, but not the findings of Leaver and Seawright, are in agreement with Poyser and Brydon (1983) who showed that oestradiol ( $10\mu\text{g/ml}$ ) had no significant effect on  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from either the day 7 or day 15 guinea-pig uterus superfused in vitro and treated with oestradiol for 3h. Poyser (1987b)

confirmed that oestradiol (1 and 10 $\mu$ g/ml) had no significant stimulatory effect on PG outputs from the superfused day 7 and day 15 guinea-pig uterus. Wilson, Liggins, Aimer, and Watkins (1986) were also unable to demonstrate a stimulatory action of oestradiol in dispersed human endometrial cells, which conflicts with previous observations using human endometrial preparations, but agrees with the findings presented here. Oestradiol (10 $\mu$ g/ml) actually inhibited the increases in PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  outputs from the day 7 and day 15 superfused guinea-pig uterus induced by the addition of arachidonic acid to the superfusing medium. Furthermore, oestradiol (10 $\mu$ g/ml) caused an inhibition of PG output from the superfused guinea-pig uterus induced by A23187 treatment (Poyser, 1987b). Oestradiol appears to have an inhibitory effect on the conversion of arachidonic acid into PGs and this may be a factor involved in the lack of stimulation of endometrial PG output. In the studies in this thesis, oestradiol (1000ng/ml) reduced PG output from guinea-pig endometrium in culture and this inhibition of endometrial PG output may also be due to an inhibition of the conversion of arachidonic acid to PGs. The inhibitory effects of high doses of oestradiol on PG synthesis and release from cultured guinea-pig endometrium in these studies are in agreement with findings in vivo. Large, non-physiological doses of oestradiol inhibit luteolysis in the guinea-pig (Illingworth and Perry, 1973), whereas smaller physiological doses of oestradiol administered at an early stage of the cycle induce premature luteolysis due to stimulation of PGF<sub>2 $\alpha$</sub>  output from the uterus (Blatchley, Donovan, Horton and Poyser, 1972).

Oestradiol does not act as a cyclo-oxygenase inhibitor, because 10 $\mu$ g/ml oestradiol stimulated PG production by homogenates of day 7 guinea-pig uteri (Naylor and Poyser, 1975). This stimulatory effect



of oestradiol on PG synthesis by broken cell preparations has also been observed in rat uterine homogenates where  $\text{PGF}_{2\alpha}$  production was stimulated by the addition of oestradiol to the incubates (Jouanen, Saintot, Thaler-Dao and Crastes de Paulet, 1985). Furthermore, oestradiol did not inhibit basal PG output from the superfused guinea-pig uterus unlike the cyclo-oxygenase inhibitor, indomethacin (Poyser, 1985a). Oestradiol may therefore prevent the access of endogenous arachidonic acid after its release to the PGH synthase enzyme for PG synthesis. This may be a direct, non-genomic effect of oestradiol. Oestradiol has been reported to induce other non-genomic effects including changes in water permeability and membrane stabilisation (Duval, Durant and Homo-Delarche, 1983).

Calmodulin synthesis in the guinea-pig uterus may also be controlled by ovarian steroids. Flandroy, Cheung and Steiner (1983) showed that oestradiol increases calmodulin levels in the rat uterus, and oestradiol and progesterone increase calmodulin concentrations in the rabbit myometrium (Matsui, Higashi, Fukunaga, Miyazaki, Maeyama and Miyamoto, 1983). A similar increase in uterine calmodulin concentrations in the guinea-pig may be required to enable the maximum stimulation of  $\text{PGF}_{2\alpha}$  synthesis to occur towards the end of the cycle. This suggestion requires further investigation.

Progesterone treatment inhibited  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs from both day 7 and day 15 guinea-pig uterus, cultured for 24h or three days. This inhibitory action of progesterone on PG synthesis and release has been previously reported to occur in day 7 and day 15 cultured guinea-pig endometrium (Leaver and Seawright, 1982), and in cultured human endometrium (Cane and Villee, 1975; Skinner, Liggins, Wilson and Neale, 1984; Wilson, Liggins, Aimer and Watkins, 1986). Although this inhibitory action of progesterone occurs during the menstrual



cycle, Tsang and Ooi (1982) demonstrated that progesterone inhibited PGF production in proliferative but not in secretory endometrium, whereas Abel and Baird (1980) using physiological doses of progesterone found a decrease in PG output from both secretory and proliferative endometrium in culture. Wilson, Liggins, Aimer and Watkins (1986) demonstrated that the inhibitory effect of progesterone on endometrial PGF<sub>2α</sub> production increased with the day of the cycle in women. However, Schatz, Markiewicz and Gurside (1985) demonstrated that, although progesterone treatment inhibited PGF<sub>2α</sub> output from both proliferative and secretory endometrium in organ culture, this inhibition was more pronounced in proliferative tissue. Clearly differences exist between these reports, and these may be due to different incubation and culture conditions. Nevertheless, the effect of progesterone on guinea-pig endometrium in culture on the inhibition of PG output appears to be similar to human endometrium, and it confirms a previous report in the guinea-pig by Leaver and Seawright (1982). Wilson, Liggins, Aimer and Watkins (1986) showed that progesterone inhibited the release of arachidonic acid (and hence inhibited PG synthesis and release) from dispersed endometrial epithelial cells. Therefore they proposed that, as the inhibitory effect occurred within 15 min and was not significantly greater after 24h than 1h, this inhibitory response to progesterone does not depend upon protein synthesis or classical receptor interaction. However, in the short term (up to 3h), progesterone (10μg/ml) had no effect on the basal outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the guinea-pig uterus superfused in vitro (Poyser and Brydon, 1983; Poyser, 1987b).

In the ovariectomised mouse, a single injection of oestradiol induces cellular proliferation of the endometrial epithelium, while having little effect on DNA synthetic activity of the endometrial

stroma (Martin, 1980). However, animals treated with progesterone for two days before administration of oestradiol show a dramatic proliferative response in the stroma, but no DNA synthetic activity in the epithelium (Martin, 1980). Studies in both the rat and mouse have shown that progesterone antagonises the synthesis of the oestrogen receptor (Hsueh, Peck and Clark, 1975), inhibits nuclear retention of the oestradiol-oestrogen receptor complex (Okulicz, Evans and Leavitt, 1981), and inhibits oestradiol uptake specifically by the endometrial epithelium (Prasad, Sar and Stumpf, 1976). Therefore, progesterone may interfere with the interaction of oestradiol and its receptor, or with the oestradiol-oestrogen receptor complex and the nucleus, and these actions may contribute to the inhibitory effect of progesterone on the stimulation of PG synthesis by oestradiol *in vivo*. Indeed, in the guinea-pig uterus, endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity was stimulated 3.2-fold by oestradiol, but when progesterone is also administered,  $\text{PGF}_{2\alpha}$  synthesizing capacity was only stimulated 1.7-fold (Poyser, 1983a). Also, endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity is highest at the end of the cycle when plasma oestradiol levels are highest and plasma progesterone levels are lowest (Poyser, 1983a).

In the studies in this thesis, treatment with progesterone and with a high dose of oestradiol (1000ng/ml) inhibited  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs, but not 6-keto- $\text{PGF}_{1\alpha}$  output, from both the day 7 and day 15 guinea-pig endometrium in culture (although 100ng progesterone/ml inhibited 6-keto- $\text{PGF}_{1\alpha}$  output from day 15 endometrium between 18h and 24h of culture). Furthermore, administration of progesterone and oestradiol (1000ng/ml) to day 7 and day 15 guinea-pig endometrium in culture inhibited the synthesizing capacities of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ . However, these treatments had only a slight inhibitory action on the

6-keto-PGF<sub>1α</sub> synthesizing capacity of day 15, but not of day 7 cultured guinea-pig endometrium. Also, treatment with progesterone and oestradiol (1000ng/ml) inhibited the synthesis of secreted proteins, but not of cellular proteins, by day 15 guinea-pig endometrium. Oestradiol (1000ng/ml) also inhibited secreted protein synthesis by day 7 endometrium. Clearly, from these results both progesterone and high doses of oestradiol have almost identical effects on PG and protein syntheses by both day 7 and day 15 guinea-pig endometrium in culture. This progesterone-like activity of oestradiol could also explain the anti-luteolytic action of high doses (non-physiological) of oestradiol reported by Illingworth and Perry (1973) in the guinea-pig.

Recently, a new family of proteins of 35-36 kDa has been reported, and these proteins have been isolated from several tissues and assigned different names. These include the calpactins I and II from bovine intestine, proteins I and II from pig intestine, lipocortins I and II from various tissues including human endometrium and placenta, calelectrin from bovine liver, calcimedin from chicken liver, cytosynalin from bovine brain, p36 from chicken embryo fibroblasts or lymphocytes, p35 from A-431 cells and chromobindins 6 and 7 from adrenal medulla (Martin, Derancourt, Capony, Watrin and Cavadore, 1988). There is much homology within this group; p.36, protein I, calpactin I and lipocortin II appear to be similar. Furthermore, p35, calpactin II and lipocortin I are also thought to be related (Haigler, Schlaepfer and Burgess, 1987; Martin, Derancourt, Capony, Watrin and Cavadore, 1988). Glenney and Tack (1985) showed that lipocortin II (p36) are cleared by limited proteolysis into two products of 3kDa and 33kDa, and the 3kDa N-terminal part contains the site of phosphorylation by C-kinase (Gould, Woodgett, Isacke and

Hunter, 1986). The complete sequences of p36 and p35 and the deduced protein sequences have shown that their proteins contain multiple domains with repeated sequences of about 70 amino acid residues.

Martin, Derancourt, Capony, Watkin and Cavadore (1988) isolated two 36kDa proteins from smooth muscle bovine aorta; one was a monomer and the other one was complexed with a 10kDa subunit protein. These were thought to be identical or at least very similar to p36 and calpactin I. The 36kDa and 10kDa were bound very strongly and this complex bound very strongly to calmodulin in a calcium-dependent manner; this binding property to calmodulin was not found in the uncomplexed 36kDa manner. Gerke and Weber (1985) reported that p36 + p10 protein complex induced formation of F-actin bundles, whereas Martin, Derancourt, Capony, Watrin and Cavadore (1988) discovered the 36kDa + 10kDa protein complex broke F-actin filaments in a  $\text{Ca}^{2+}$ -dependent manner, and that there was no obvious reason for this difference. However, from these data, it was suggested that the 36kDa + 10kDa protein complex may modulate some  $\text{Ca}^{2+}$ -regulated effects mediated by calmodulin. So, the action of these two homologous proteins on  $\text{PLA}_2$  would appear to be primarily inhibitory, via inhibiting calmodulin, and hence PG synthesis should be reduced. What are the implications of these findings for PG synthesis by the uterus ?

In cultured human endometrium, the glucocorticoid hormone, dexamethasone, stimulates the production of lipocortins by both proliferative and secretory endometrium (Gurpide, Markiewicz, Schatz and Hirata, 1986). However, Schatz, Markiewicz and Gurpide (1986) reported that dexamethasone treatment only inhibited  $\text{PGF}_{2\alpha}$  production by secretory endometrium. Kelly and Smith (1987) found only a small inhibitory effect of dexamethasone on  $\text{PGF}_{2\alpha}$  production by

proliferative endometrium. Treatment with hydrocortisone, the main glucocorticoid hormone secreted by the guinea-pig (Fajer and Vogt, 1963) had no effect on the outputs  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the cultured day 15 guinea-pig endometrium. Furthermore, hydrocortisone ( $10\mu\text{g/ml}$ ) treatment of the day 15 guinea-pig uterus superfused in vitro for 3h had no effects on either the basal or A23187-stimulated outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$  (Poyser, 1987b). TPA activates protein kinase C which in turn phosphorylates lipocortin, thus inactivating it and removing its inhibitory influence on  $\text{PLA}_2$  activity and, hence, on PG synthesis (Nishizuka, 1986). The administration of TPA to day 7 and day 15 cultured guinea-pig endometrium had no significant effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ . Also, TPA had no effect on either basal or A23187-stimulated PG outputs from the day 7 and day 15 guinea-pig uterus superfused in vitro (Poyser 1987a). The results of Poyser (1987a, b) are consistent with the findings in these studies that hydrocortisone and protein kinase C have no effect on PG output from the guinea-pig endometrium. Therefore, by inference, lipocortin and related proteins are not a controlling factor in PG synthesis and release from the guinea-pig endometrium.

In the studies presented in this thesis, the protein and RNA synthesis inhibitors actinomycin D, cycloheximide and puromycin have been demonstrated to inhibit  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  release from cultured day 7 and day 15 guinea-pig endometrium at concentrations greater than  $1\mu\text{g/ml}$ . Smith and Kelly (1987) have also examined the effect of actinomycin D on PGF and PGE production by glandular and stromal cells from human endometrium in the secretory phase. They showed that actinomycin D ( $100\text{ng/ml}$ ) had no effect on basal or oestradiol-stimulated PGF output and PGE output from either glandular

or stromal cells. Paradoxically, Smith and Kelly (1987) observed a stimulation of PGF and PGE outputs with actinomycin D treatment. They suggested from these findings that basal release of PGF and PGE from secretory glandular epithelial cells from the human endometrium is suppressed and that inhibition of protein synthesis reverses this effect. However, Smith and Kelly (1987) did not demonstrate that this dose of actinomycin D (100ng/ml) inhibited protein synthesis, unlike the higher doses used in this study which did inhibit both secreted and cellular protein synthesis and also uterine PG production.

Treatment with intra-uterine actinomycin D in vivo on day 10 inhibited the basal outputs of PGF<sub>2α</sub> and PGE<sub>2</sub> but not 6-keto-PGF<sub>1α</sub>, from the guinea-pig uterus when measured in vitro on day 15. Castracane and Jordan (1976) treated ovariectomised rats after progesterone priming with a single dose of oestradiol-17β and observed a stimulation of uterine PGF and PGE output. However, the systemic treatment of these rats with actinomycin D or cycloheximide failed to inhibit the oestrogen-stimulated uterine PG synthesis. They proposed that the action of oestradiol on the stimulation of uterine PG production was not due to stimulation of protein synthesis. However, in a previous study Poyser (1979) demonstrated that systemic treatment of guinea-pigs with actinomycin D (20μg) failed to inhibit uterine PG synthesizing capacity or extend the oestrous cycle, whereas intra-uterine actinomycin D treatment (10μg/uterine horn,; i.e. the same dose) reduced PG synthesizing capacity, prevented luteal regression and extended the cycle length. These studies have demonstrated that the effect of intra-uterine actinomycin D treatment is to inhibit PG synthesis by and release from the guinea-pig uterus. Therefore, the study by Castracane and Jordan (1976) should be repeated, but the

protein synthesis inhibitors need to be administered directly into the uterus and not systemically in order to examine their effect on uterine PG synthesis. Overall, the studies presented in this thesis indicate that increased protein synthesis is necessary for the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis in the guinea-pig.

Recently, proteins have been implicated in the control of PG production in several different cell types, because inhibitors of protein synthesis have been demonstrated to inhibit PG synthesis. These include:  $\text{PGE}_2$  synthesis by transformed fibroblasts in response to serum, thrombin and bradykinin is inhibited by actinomycin D and cycloheximide (Pong, Hong and Levine, 1977);  $\text{PGE}_2$  synthesis by skeletal muscle is inhibited by puromycin and emetine (Turinsky, 1985);  $\text{PGI}_2$  synthesis by endothelial cells, stimulated by bradykinin and leukotrienes, is inhibited by cycloheximide and actinomycin D (Clark, Littlejohn, Mong and Crooke, 1986);  $\text{PGI}_2$  and  $\text{PGE}_2$  synthesis by macrophages stimulated by TPA and zymosan is inhibited by actinomycin D (Bonney, Wightman, Dahlgren, Davies, Kuehl and Humes, 1980); and  $\text{PGE}_2$  synthesis by the brain, stimulated by endogenous pyrogen, is inhibited by anisomycin (Townsend, Cranston and Hellon, 1984).

There has been several reports of proteins that exert a stimulatory effect on PG synthesis. Subbiah, Yunker, Yamamoto, Kottke and Bale (1985) demonstrated that PG synthesis in cultured skin fibroblasts was stimulated by human breast milk. Subsequently, Bydlowski, Yunker, Campaigne, Kotogal and Subbiah (1986) showed that human breast milk contained a protein of less than 10kDa which had a potent stimulatory effect on  $\text{PGI}_2$  synthesis (measured as 6-keto- $\text{PGF}_{1\alpha}$ ) from rabbit aorta. A protein or protein-associated substance of less than 10kDa in human fetal urine induced a 10- to 600-fold increase in



PGE<sub>2</sub> synthesis from human amnion cells in monolayer culture (Casey, MacDonald and Mitchell, 1983). Melittin is a phospholipase A<sub>2</sub> stimulatory peptide (2,800Da) isolated from bee venom (Haberman, 1972). Bee, wasp and hornet venom peptides have a well characterised ability to stimulate PLA<sub>2</sub> activity (Mollay and Kreil, 1974; Blackwell, Carnuccio, DiRosa, Flower, Parente and Persico, 1980). Melittin has also been demonstrated to cause an increase in intracellular Ca<sup>2+</sup> concentrations in cultured human fibroblasts, an effect which is inhibited by TMB-8. This suggests that melittin may exert its PLA<sub>2</sub> stimulatory action by releasing calcium from intracellular stores (Mix, Dinerstein and Villereal, 1984). Subsequently, Clark, Conway, Shorr and Crooke (1987) prepared antibodies against melittin and the antibodies were used to identify a mammalian protein (in murine smooth muscle line BC3H<sub>1</sub>) with similar functions and antigenic properties. This protein (28kDa) was termed 'phospholipase A<sub>2</sub> - activating protein' and selectively stimulated PLA<sub>2</sub> activity in cell-free sonicates of the smooth muscle cell line, but had no effect on PLC or on pancreatic or snake venom PLA<sub>2</sub>. Clark, Conway, Shorr and Crooke (1987) also found the phospholipase A<sub>2</sub> - activating protein in the bovine endothelial cell line CPAE, the human monocytic cell line U937, and the murine T-cell line E24. Clark, Chen, Crooke and Bomalski (1988) demonstrated that tumour necrosis factor induced [<sup>3</sup>H]-arachidonic acid metabolite release from bovine endothelial CPAE cells, an effect apparently mediated by a transient increase in PLA<sub>2</sub> activity. This action was prevented by cycloheximide (100µg/ml) and actinomycin D (10µg/ml) treatment showing that protein and RNA synthesis is required for PLA<sub>2</sub> activation. They also showed that the amount of the phospholipase A<sub>2</sub> - activating protein increased following administration of tumour necrosis factor



treatment, and that the time-course of induction of the phospholipase A<sub>2</sub> - activating protein correlated with the induction of PLA<sub>2</sub> activity. Clearly, this protein with phospholipase A<sub>2</sub> stimulatory activity may be found in many different cells, and PLA<sub>2</sub> activity may not only be inhibited but also stimulated by regulatory proteins. It is also interesting to speculate whether the phospholipase A<sub>2</sub> - activating protein stimulates the release of Ca<sup>2+</sup> from intracellular stores like melittin, and whether a "phospholipase A<sub>2</sub> -activating protein-like factor" is involved in the stimulation of PGF<sub>2</sub>α production by the guinea-pig endometrium towards the end of the oestrous cycle.

One of the main mechanisms by which hormones and neurotransmitters elicit an intracellular response is by binding to a cell surface receptor linked to membrane-bound, GTP-binding proteins (G-proteins). These G-proteins transduce the "ligand-receptor binding signals" to membrane associated effector systems that produce second messengers which control the required intracellular action. G-proteins transduce receptor-mediated signals for a variety of effector systems, including cyclic nucleotide phosphodiesterases, adenylate cyclase, phospholipase C and ion channels (Gilman, 1987). Recently, G-proteins have also been implicated in the activation of PLA<sub>2</sub>. For example, in the rod outer segments of bovine retina, light activation of PLA<sub>2</sub> has been demonstrated to occur by a transducin-dependent mechanism, with transducin the major G-protein in this preparation (Jelsema and Axelrod, 1987). G-proteins have also been implicated in the activation of PLA<sub>2</sub> and in the release of arachidonic acid in cells of the rat thyroid cell line FRTL5 in response to α<sub>1</sub>-adrenergic stimulation (Burch, Luini and Axelrod, 1986). Burch and Axelrod (1987) have proposed that stimulation of Swiss 3T3 fibroblasts

results in arachidonic acid release by PLA<sub>2</sub> and this response is mediated via a G-protein. However, it appears unlikely that PLA<sub>2</sub> in the guinea-pig uterus is stimulated by G-proteins, since they are primarily concerned with eliciting a rapid response mediated by a membrane-bound receptor. Stimulation of uterine PG production in the guinea-pig is controlled over several days in responses to intracellular responses via nuclear steroid receptors which mediate the synthesis of protein(s) which stimulates PLA<sub>2</sub>.

If pregnancy occurs, the luteolytic effect of the uterus must be inhibited, because ovarian progesterone is required in the guinea-pig for the maintenance of the first 4-5 weeks of pregnancy (Heap and Deanesly, 1966; Csapo, Puri and Tarro, 1981). In the non-pregnant guinea-pig, oestradiol output from the guinea-pig ovary increases after 10 day of the cycle (Joshi, Watson and Labhsetwar, 1973), just before the increase in uterine PGF<sub>2α</sub> output measured in the utero-ovarian venous plasma (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975). However, if the guinea-pig is pregnant, the increase in ovarian oestradiol and uterine PGF<sub>2α</sub> outputs after day 10, as measured in the utero-ovarian plasma, do not occur and plasma progesterone levels remain high (Blatchley, Maule Walker and Poyser, 1975a, b; Antonini, Turner and Pauerstein, 1976). Henderson, Scaramuzzi and Baird (1977) showed that PGE<sub>2</sub> delayed luteal regression caused by PGF<sub>2α</sub> in sheep, and therefore PGE<sub>2</sub> was proposed as a putative luteotrophic factor. However, in the guinea-pig, Antonini, Turner and Pauerstein (1977) demonstrated that PGE<sub>2</sub> output from the ovary was not increased during this early stage of pregnancy, and this finding was confirmed by Poyser (1984b). Consequently, PGE<sub>2</sub> is not involved in luteal maintenance in the guinea-pig.

In the sheep, the mechanism by which the conceptus prevents the

luteolytic effect of  $\text{PGF}_{2\alpha}$  is becoming clearer. A polypeptide of 17kDa has been isolated as the major secretory protein produced by the conceptus between days 13 and 21 of pregnancy (Godkin, Bazer, Moffat, Sessions and Roberts, 1982). This protein (ovine trophoblast protein - one; oTP-1) bound to receptors present both in the endometrium and in the corpus luteum (Godkin, Bazer, Thatcher and Roberts, 1984). However, oTP-1 did not stimulate luteal progesterone production (Godkin, Bazer and Roberts, 1984) and this suggested that oTP-1 was eliciting its anti-luteolytic effect on the endometrium. Godkin, Bazer and Roberts (1984) also observed that oTP-1 stimulated the synthesis of several endometrial proteins. Proteins released by the sheep conceptus in culture and injected into non-pregnant sheep suppressed the induction of uterine  $\text{PGF}_{2\alpha}$  release by oestradiol and oxytocin (Fincher, Bazer, Hansen, Thatcher and Roberts, 1986). Subsequently, Vallet, Bazer and Roberts (1987) showed that oTP-1 induced both an increase in secretion of some proteins and a decrease in the secretion of other proteins by sheep endometrial explants. The origin of these endometrial proteins was not determined, although it was speculated that they were produced by surface and upper glandular epithelial uterine layers, since oTP-1 receptors have been localised in these layers by immunocytochemical techniques (Godkin, Bazer and Roberts, 1984). These glandular epithelial sites are also the sites of  $\text{PGF}_{2\alpha}$  synthesis. The protein oTP-1 may induce the production by the endometrium of a luteotrophic factor, as suggested by Vallet, Bazer and Roberts (1987), but it may also inhibit the production of a  $\text{PLA}_2$  stimulatory protein in the uterus that is required for the increase in  $\text{PGF}_{2\alpha}$  production at the end of the cycle. A similar "oTP-1 like" protein is produced by the bovine conceptus and is a major product released from these conceptuses between day 16-26

of pregnancy, during the critical period at which the corpus luteum has to be protected from the luteolytic action of uterine  $\text{PGF}_{2\alpha}$  (Bartol, Roberts, Bazer, Lewis, Godkin and Thatcher, 1985). This protein is produced at a similar stage of development of the conceptus in the cow when compared to the sheep conceptus and is immunologically related to oTP-1, and so it has been termed bovine trophoblast protein-one (bTP-1; Helmer, Hansen, Anthony, Thatcher, Bazer and Roberts, 1987). Therefore, the cow and sheep appear to have similar mechanisms of preventing luteal regression. It may be speculated that a trophoblast protein is secreted by the guinea-pig that has a functionally similar role in the maternal recognition of pregnancy, and may control the synthesis of endometrial proteins that regulate  $\text{PLA}_2$  activity and hence PG synthesis. Certainly, there is much evidence that the guinea-pig conceptuses secrete an antiluteolytic factor which reduces endometrial  $\text{PGF}_{2\alpha}$  synthesis (Horton and Poyser, 1976; Poyser, 1984c).

Overall, the studies in this thesis have shown that  $\text{PGF}_{2\alpha}$  production in the guinea-pig endometrium is dependent on calcium and protein synthesis. It is proposed that oestradiol acting on a progesterone-primed uterus stimulates the synthesis of a protein(s) which, with  $\text{Ca}^{2+}$  mediates the increased release of arachidonic acid by  $\text{PLA}_2$  for the selective synthesis of  $\text{PGF}_{2\alpha}$  by the guinea-pig endometrium towards the end of the oestrous cycle. It is possible that, during early pregnancy, a "trophoblast protein", secreted by the guinea-pig conceptuses, prevents the synthesis of this  $\text{PLA}_2$ -stimulating protein. Increased  $\text{PGF}_{2\alpha}$  synthesis is therefore prevented and the corpora lutea are maintained.

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4. Effects of oestradiol, progesterone, hydrocortisone and oxytocin on prostaglandin output from the guinea-pig endometrium maintained in tissue culture. Prostaglandins (1987) 34, 535-551. S.C. Riley and N.L. Poyser.

## EFFECTS OF OESTRADIOL, PROGESTERONE, HYDROCORTISONE AND OXYTOCIN ON PROSTAGLANDIN OUTPUT FROM THE GUINEA-PIG ENDOMETRIUM MAINTAINED IN TISSUE CULTURE.

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### ABSTRACT

The effects of oestradiol, oxytocin, progesterone and hydrocortisone *in vitro* on prostaglandin (PG) output from guinea-pig endometrium, removed on days 7 and 15 of the oestrous cycle and maintained in tissue culture for 3 days, have been investigated. Oestradiol (3.7 to 3700nM) and oxytocin (2 to 200pM) did not stimulate endometrial PGF<sub>2α</sub> output, thus not confirming the findings of a previous report (Leaver & Seawright, 1982), nor did they stimulate the outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>. In fact, oestradiol (3700nM) inhibited the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and, to a lesser extent, 6-keto-PGF<sub>1α</sub>. Progesterone (3.2 to 3200nM) inhibited the outputs of PGF<sub>2α</sub> and PGE<sub>2</sub>; hydrocortisone (2.8 to 2800nM) had no effect on endometrial PG output. These findings indicate that the inhibitory effect of progesterone on endometrial PG synthesis and release in the guinea-pig is not due to progesterone having a glucocorticoid-like action. Furthermore, progesterone had no effect on 6-keto-PGF<sub>1α</sub> output, suggesting that the mechanisms controlling endometrial PGI<sub>2</sub> synthesis (as reflected by measuring 6-keto-PGF<sub>1α</sub>) are different from those controlling endometrial PGF<sub>2α</sub> and PGE<sub>2</sub> synthesis.

### INTRODUCTION

Many studies indicate that prostaglandin (PG) F<sub>2α</sub> is the uterine luteolytic hormone in non-primate mammalian species (see 1,2), including the guinea-pig (3). The output of PGF<sub>2α</sub> from the guinea-pig uterus increases from day 11 of the oestrous cycle (4,5,6). Oestradiol acting on a progesterone-primed uterus appears responsible for this increase, since treatment of ovariectomized guinea-pigs with these two steroid hormones causes a large increase in uterine PGF<sub>2α</sub> output (7), and oestradiol output from the guinea-pig ovary increases after day 10 of the cycle (8). However, studies in several mammalian species have shown that the maximum output of PGF<sub>2α</sub> from the uterus does not occur until after



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plasma progesterone levels have started to fall (see 1,2), indicating that progesterone has inhibitory as well as facilitatory effects on uterine  $\text{PGF}_{2\alpha}$  production. Indeed, basal and oestradiol-stimulated outputs of  $\text{PGF}_{2\alpha}$  from human and guinea-pig endometrium maintained in tissue culture, and from human endometrial cells maintained in cell culture are inhibited by progesterone treatment in vitro (9,10,11,12). Glucocorticoid hormones stimulate the production by many tissues of lipocortin, a protein which inhibits phospholipase  $\text{A}_2$  [EC. 3.1.1.4] activity and thereby decreases PG synthesis and release by reducing the supply of substrate (see 13). Consequently, the question is raised as to whether progesterone inhibits  $\text{PGF}_{2\alpha}$  synthesis by the endometrium due to a glucocorticoid-like action. The purpose of this study was to compare the effects of progesterone and hydrocortisone, the main glucocorticoid hormone in the guinea-pig (14), on basal PG output and on the previously reported (15) stimulation of  $\text{PGF}_{2\alpha}$  output by oestradiol and oxytocin from guinea-pig endometrium maintained in tissue culture.

### METHODS

Female, virgin guinea-pigs weighing between 650 and 950g were examined daily and a vaginal smear was taken when the vaginal membrane was open. The first day of the oestrous cycle was designated as the day before the post-ovulatory influx of leucocytes when cornification is at a maximum. All animals had normal cycles before being killed, by stunning and incising the neck, on day 7 or day 15 of the cycle. The uterus from each guinea-pig was removed and placed in culture medium. The endometrium was dissected away from the myometrium by cutting away 1 x 2mm pieces of endometrium, under sterile conditions. Approximately 12mg endometrium was placed on a raised platform in a Petri dish, which contained 4ml Medium 199 plus Earle's salts, supplemented with glutamine and antibiotics (16). Eighteen Petri dishes containing endometrial tissue were prepared from each guinea-pig; 14 of the dishes contained "hormonal treatments" (performed in duplicate) as outlined below and four dishes were untreated (controls). The Petri dishes were placed in two modified Kilner jars (nine dishes per jar) such that each set of nine dishes contained one of the duplicate treatments and two controls. The endometrium was cultured for 72h as described previously, and the tissue remains viable during this period (16,17). The culture medium was changed every 24h. The samples of culture medium were stored at  $-20^\circ\text{C}$  before radioimmunoassay, without extraction, for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ . There were 5 animals per group which produced 10 samples per time point.

### Hormonal Treatments

Experiment 1 Day 7 and day 15 guinea-pig endometrium (i.e. endometrium removed from guinea-pigs on days 7 and 15 of the



oestrous cycle respectively) was treated on day 2 (24 to 48h period) of culture with the following compounds: oestradiol-17 $\beta$  (37, 370 and 3700nM); progesterone (32 and 320nM); oxytocin (20 and 200pM).

Experiment 2 a. Day 15 guinea-pig endometrium was treated on all 3 days of culture with the following compounds: oestradiol-17 $\beta$  (3.7, 37, 370 and 3700nM); progesterone (3.2, 32, 320 and 3200nM); hydrocortisone (2.8, 28, 280 and 2800nM); oxytocin (2, 20 and 200pM).

b. Day 7 guinea-pig endometrium was treated on all 3 days of culture with oestradiol-17 $\beta$ ; (3.7, 37, 370 and 3700nM).

Sources of Materials Culture medium, glutamine and antibiotics were obtained from Flow Laboratories, Rickmansworth, Herts., U.K.; sterile Petri dishes from Sterilin Ltd., Teddington, Middlesex, U.K.; oestradiol-17 $\beta$ , progesterone, hydrocortisone and oxytocin from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Details of Radioimmunoassay Prostaglandin F $_{2\alpha}$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  were measured using antibodies raised in this laboratory and whose cross-reactivities have been reported elsewhere (18,19,20,21). The only significant cross-reactivities are PGF $_{1\alpha}$  (100%) with the PGF $_{2\alpha}$  antiserum, and PGE $_1$  (66%), PGA $_2$  (25.5%) and PGB $_2$  (11.8%) with the PGE $_1$  antiserum. Previous studies involving analysis by gas chromatography and mass spectrometry have shown that the guinea-pig uterus synthesises very little PGF $_{1\alpha}$  in comparison to PGF $_{2\alpha}$ , and that there is no detectable synthesis of PGE $_1$ , PGA $_2$  and PGB $_2$  (22). Therefore, it is likely that the PGF $_{2\alpha}$  and PGE $_2$  antisera were measuring predominantly the PG to which they had been raised. The inter-assay coefficients of variation were 8.2% (PGF $_{2\alpha}$ ), 8.6% (PGE $_2$ ), and 6.6% (6-keto-PGF $_{1\alpha}$ ). The intra-assay coefficients of variation were 5.6% (PGF $_{2\alpha}$ ), 7.8% (PGE $_2$ ), and 5.9% (6-keto-PGF $_{1\alpha}$ ). The limits of detection were 30pg PGF $_{2\alpha}$ , 40pg PGE $_2$  and 30pg 6-keto-PGF $_{1\alpha}$  per assay tube.

Statistical tests Results were analysed by Student's t-test.

## RESULTS

The outputs of PGF $_{2\alpha}$ , PGE $_2$  and 6-keto-PGF $_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium are shown in Figure 1. PGF $_{2\alpha}$  is the major PG released from day 15 guinea-pig endometrium, together with lesser quantities of PGE $_2$  and 6-keto-PGF $_{1\alpha}$ . On day 7, endometrial PGF $_{2\alpha}$  and 6-keto-PGF $_{1\alpha}$  outputs are similar while PGE $_2$  output is somewhat lower. The outputs of PGF $_{2\alpha}$ , PGE $_2$  and, to a limited extent, 6-keto-PGF $_{1\alpha}$  are higher from day 15 than from day 7 endometrium, and the output of all 3 PGs falls during the 3-day period of culture.

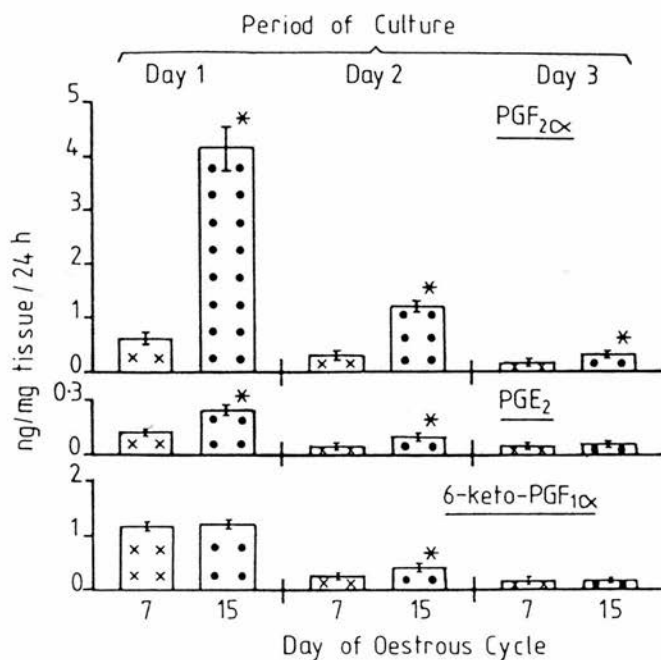


FIGURE 1. Mean ( $\pm$  s.e.m.,  $n = 10$ ) outputs of prostaglandin (PG)  $\text{F}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from guinea-pig endometrium, removed on days 7 and 15 of the oestrous cycle, on days 1, 2 and 3 of tissue culture.

\* Significantly ( $P < 0.05$ ) higher than day 7 value for same PG on same day of culture (Student's  $t$ -test).

Experiment 1. The addition of oestradiol (37 to 3700nM) or oxytocin (20 and 200pM) to the culture medium only on day 2 of culture had no stimulatory effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium (Tables 1, 2 and 3). In fact, oestradiol at the highest concentration (3700nM) significantly ( $P < 0.05$ ) inhibited the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and, except for 6-keto- $\text{PGF}_{1\alpha}$ , from day 15 guinea-pig endometrium on day 3 of culture. Progesterone (320nM) added to the culture medium only on day 2 of culture significantly ( $P < 0.05$ ) reduced (i)  $\text{PGF}_{2\alpha}$  output from day 7 endometrium on day 2 of culture, (ii)  $\text{PGF}_{2\alpha}$  output from day 15 endometrium on day 3 of culture, and (iii)  $\text{PGE}_2$  output from day 7 and day 15 endometrium on day 2 of culture (Tables 1 and 2). Progesterone (32nM) significantly ( $P < 0.05$ ) reduced  $\text{PGF}_{2\alpha}$  output from day 7 guinea-pig endometrium on day 2 of culture (Table 2). The outputs of 6-keto- $\text{PGF}_{1\alpha}$  were unaffected by progesterone treatment (Table 3).

Experiment 2. The addition of oestradiol (3.7 to 3700nM) or oxytocin (2 to 200pM) to the culture medium for all 3 days of culture had no stimulatory effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium (Tables 4, 5 and 6). At the highest concentration (3700nM), oestradiol significantly ( $P < 0.05$ ) reduced the output of  $\text{PGF}_{2\alpha}$  from day 7 and day 15 endometrium on all 3 days of culture (Table 4). Similarly, oestradiol (3700nM) significantly ( $P < 0.05$ ) reduced the output of  $\text{PGE}_2$  from day 15 endometrium on days 2 and 3 of culture, and from day 7 endometrium on days 1 and 2 of culture (Table 5). (On day 3 of culture, the output of  $\text{PGE}_2$  from progesterone-treated day 7 endometrium was below the detection limit of 10pg/mg tissue/24h, and was therefore reduced). Oestradiol (3700nM) had no significant inhibitory effect on 6-keto- $\text{PGF}_{1\alpha}$  output, although the output of 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium tended to be reduced on days 2 and 3 of culture by this treatment (Table 6).

The addition of progesterone (3.2 to 3200nM) to the culture medium for 3 days significantly ( $P < 0.05$ ) reduced the output of  $\text{PGF}_{2\alpha}$  from day 15 endometrium on day 1 of culture (except for 3.2nM), on day 2 of culture, and on day 3 of culture (3200nM only; Table 4). The output of  $\text{PGE}_2$  from day 15 endometrium was significantly ( $P < 0.05$ ) reduced by progesterone (320nM) on day 2 of culture, and by progesterone (3200nM) on days 1 and 2 of culture (Table 5). The output of 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium was unaffected by progesterone treatment (Table 6).

Hydrocortisone (2.8 to 2800nM) had no effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 to guinea-pig endometrium when present in the culture medium for all 3 days (Tables 4, 5 & 6).

Table 1. Mean ( $\pm$  s.e.m.,  $n = 10$ ) output of prostaglandin (PG) F<sub>2α</sub> (expressed as % of day 1 value) from cultured guinea-pig endometrium, removed on days 7 and 15 of the oestrous cycle, and not treated (Control) or treated with oestradiol, oxytocin and progesterone on day 2 of culture.

		Output of PGF <sub>2α</sub> (% of value on day 1 of culture)					
Day of cycle:		7			15		
Day of culture:		1	2	3	1	2	3
Treatment:							
Control		100	51.1 $\pm$ 6.2	26.1 $\pm$ 6.0	100	25.9 $\pm$ 3.8	8.0 $\pm$ 1.6
Oestradiol:							
37nM		100	56.2 $\pm$ 5.9	20.4 $\pm$ 3.9	100	19.6 $\pm$ 2.3	6.6 $\pm$ 0.9
370nM		100	45.2 $\pm$ 5.5	28.0 $\pm$ 5.2	100	22.6 $\pm$ 2.7	6.2 $\pm$ 1.0
3700nM		100	38.5 $\pm$ 3.9	11.5 $\pm$ 1.7 *	100	20.9 $\pm$ 2.6	4.0 $\pm$ 0.7 *
Oxytocin:							
20pM		100	57.9 $\pm$ 9.9	27.7 $\pm$ 7.5	100	23.8 $\pm$ 3.4	7.6 $\pm$ 1.8
200pM		100	50.7 $\pm$ 6.1	27.7 $\pm$ 4.3	100	23.8 $\pm$ 2.9	7.2 $\pm$ 1.7
Progesterone:							
32nM		100	34.7 $\pm$ 3.1 *	18.7 $\pm$ 2.4	100	27.8 $\pm$ 4.4	4.5 $\pm$ 0.8
320nM		100	30.4 $\pm$ 2.7 *	16.2 $\pm$ 3.1	100	24.3 $\pm$ 2.2	3.9 $\pm$ 0.8 *

\* significantly ( $P < 0.05$ ) lower than corresponding control value on same day of cycle and same day of culture

Table 2. Mean ( $\pm$  s.e.m.,  $n = 10$ ) output of prostaglandin (PG) E<sub>2</sub> (expressed as % of day 1 value) from cultured guinea-pig endometrium, removed on days 7 and 15 of the oestrous cycle, and not treated (Control) or treated with oestradiol, oxytocin and progesterone on day 2 of culture.

Output of PGE <sub>2</sub> $\alpha$ (% of value on day 1 of culture)						
Day of cycle:	7			15		
Day of culture:	1	2	3	1	2	3
Treatment:						
Control	100	32.2 $\pm$ 4.9	21.4 $\pm$ 4.1	100	31.2 $\pm$ 4.0	9.4 $\pm$ 1.7
Oestradiol:						
37nM	100	38.1 $\pm$ 5.2	22.2 $\pm$ 3.8	100	25.6 $\pm$ 2.8	8.0 $\pm$ 1.5
370nM	100	31.3 $\pm$ 4.5	28.5 $\pm$ 6.4	100	25.2 $\pm$ 3.5	7.5 $\pm$ 1.1
3700nM	100	25.5 $\pm$ 4.9	10.2 $\pm$ 1.5 *	100	21.4 $\pm$ 3.1	4.3 $\pm$ 0.9 *
Oxytocin:						
20pM	100	29.7 $\pm$ 4.2	19.7 $\pm$ 3.5	100	26.7 $\pm$ 2.9	5.9 $\pm$ 1.0
200pM	100	33.5 $\pm$ 5.4	27.6 $\pm$ 7.4	100	25.4 $\pm$ 3.5	7.7 $\pm$ 1.8
Progesterone:						
32nM	100	23.3 $\pm$ 2.6	17.3 $\pm$ 3.8	100	31.6 $\pm$ 5.2	5.9 $\pm$ 0.6
320nM	100	18.6 $\pm$ 2.9 *	12.2 $\pm$ 2.9	100	21.7 $\pm$ 1.8 *	5.3 $\pm$ 1.1

\* significantly ( $\bar{p} < 0.05$ ) lower than corresponding control value on same day of cycle and same day of culture

Table 3. Mean ( $\pm$  s.e.m.,  $n = 10$ ) output of 6-keto-prostaglandin (PG)  $F_{1\alpha}$  (expressed as % of day 1 value) from cultured guinea-pig endometrium, removed on days 7 and 15 of the oestrous cycle, and not treated (Control) or treated with oestradiol, oxytocin and progesterone on day 2 of culture.

Output of 6-keto-PGF <sub>1α</sub> (% of value on day 1 of culture)						
Day of cycle:	7			15		
Day of culture:	1	2	3	1	2	3
Treatment:						
Control	100	20.8 ± 1.1	10.8 ± 0.6	100	27.5 ± 1.9	12.5 ± 1.5
Oestradiol:						
37nM	100	22.2 ± 1.9	11.3 ± 1.3	100	29.2 ± 3.6	11.4 ± 1.2
370nM	100	22.4 ± 2.5	10.4 ± 0.9	100	23.4 ± 1.4	11.0 ± 0.7
3700nM	100	21.3 ± 1.5	7.9 ± 0.5 *	100	23.8 ± 2.1	9.3 ± 0.7
Oxytocin:						
20pM	100	19.8 ± 1.3	11.0 ± 1.0	100	27.2 ± 2.3	9.6 ± 1.0
200pM	100	19.4 ± 1.6	10.0 ± 0.9	100	28.0 ± 2.5	11.2 ± 1.2
Progesterone:						
32nM	100	19.9 ± 1.2	9.3 ± 0.9	100	28.9 ± 2.4	9.7 ± 1.0
320nM	100	18.4 ± 1.6	8.6 ± 0.5	100	29.1 ± 2.5	9.5 ± 0.8

\* significantly ( $P < 0.05$ ) lower than corresponding control value on same day of cycle and same day of culture

Table 4. Mean ( $\pm$  s.e.m.,  $n = 10$ ) output of  $\text{PGF}_2\alpha$  (expressed as % of day 1 control (C) value) from day 7 and day 15 (as indicated) cultured guinea-pig endometrium and not treated (C) or treated (T) with oestradiol, oxytocin, progesterone and hydrocortisone on all 3 days of culture.

Day of culture:		Output of $\text{PGF}_2\alpha$ (% of control on day 1 of culture)					
Treatment:		1		2		3	
		C	T	C	T	C	T
Oestradiol (day 15)							
3.7nM	100	120.2 $\pm$ 8.9		40.8 $\pm$ 5.7	47.6 $\pm$ 6.3	20.7 $\pm$ 5.5	24.2 $\pm$ 7.0
37nM		106.8 $\pm$ 9.6			40.3 $\pm$ 5.5		15.2 $\pm$ 2.9
370nM		108.2 $\pm$ 11.5			38.8 $\pm$ 5.1		19.4 $\pm$ 5.3
3700nM		62.0 $\pm$ 3.0 *			13.8 $\pm$ 1.8 *		3.5 $\pm$ 0.8 *
Oestradiol (day 7)							
3.7nM	100	106.9 $\pm$ 15.5		70.2 $\pm$ 11.6	73.6 $\pm$ 14.6	29.9 $\pm$ 5.5	28.8 $\pm$ 7.6
37nM		110.8 $\pm$ 10.6			82.7 $\pm$ 16.8		29.2 $\pm$ 7.9
370nM		95.6 $\pm$ 6.5			75.1 $\pm$ 17.9		25.3 $\pm$ 5.6
3700nM		54.1 $\pm$ 5.6 *			17.5 $\pm$ 3.7 *		9.1 $\pm$ 1.7 *
Oxytocin (day 15)							
2pM	100	91.3 $\pm$ 9.9		26.7 $\pm$ 4.0	18.9 $\pm$ 2.8	4.3 $\pm$ 0.8	3.0 $\pm$ 0.5
20pM		104.4 $\pm$ 11.4			21.2 $\pm$ 3.4		3.8 $\pm$ 0.7
200pM		89.9 $\pm$ 6.8			20.5 $\pm$ 2.2		3.3 $\pm$ 0.5
Progesterone (day 15)							
3.2nM	100	84.7 $\pm$ 10.1		26.7 $\pm$ 4.0	15.0 $\pm$ 1.9 *	4.3 $\pm$ 0.8	2.9 $\pm$ 0.4
32nM		67.5 $\pm$ 6.9 *			9.0 $\pm$ 1.4 *		2.5 $\pm$ 0.7
320nM		74.1 $\pm$ 8.6 *			13.4 $\pm$ 1.6 *		2.7 $\pm$ 0.5
3200nM		69.3 $\pm$ 7.8 *			10.9 $\pm$ 1.8 *		2.0 $\pm$ 0.4 *
Hydrocortisone (day 15)							
2.8 nM	100	108.7 $\pm$ 11.8		28.4 $\pm$ 3.1	32.6 $\pm$ 6.0	6.8 $\pm$ 1.1	7.8 $\pm$ 2.0
28nM		129.4 $\pm$ 17.6			40.0 $\pm$ 6.4		9.3 $\pm$ 2.5
280nM		107.2 $\pm$ 12.8			33.0 $\pm$ 6.5		6.7 $\pm$ 1.9
2800nM		88.2 $\pm$ 8.9			24.8 $\pm$ 2.9		4.7 $\pm$ 0.5

\* significantly ( $P < 0.05$ ) lower than corresponding (C) value on same day of culture.

Table 5. Mean ( $\pm$  s.e.m.,  $n = 10$ , unless otherwise indicated) output of PGE<sub>2</sub> (expressed as % of day 1 control (C) value) from day 7 and day 15 (as indicated) cultured guinea-pig endometrium and not treated (C) or treated (T) with oestradiol, oxytocin, progesterone and hydrocortisone on all 3 days of culture.

Day of culture:	Output of PGE <sub>2</sub> (% of control on day 1 of culture)					
	1		2		3	
Treatment:	C	T	C	T	C	T
Oestradiol (day 15)						
3.7nM	100	110.4 $\pm$ 8.0	48.6 $\pm$ 5.6	55.8 $\pm$ 7.0	28.7 $\pm$ 5.1	33.7 $\pm$ 7.5
37nM		103.1 $\pm$ 12.5		55.0 $\pm$ 6.7		29.1 $\pm$ 8.7
370nM		125.7 $\pm$ 17.7		43.9 $\pm$ 5.4		23.6 $\pm$ 5.7
3700nM		100.6 $\pm$ 13.0		17.0 $\pm$ 2.4*		7.3 $\pm$ 1.7*
Oestradiol (day 7)						
3.7nM	100	113.3 $\pm$ 13.1	48.5 $\pm$ 16.0#	55.5 $\pm$ 10.0#	25.8 $\pm$ 7.6	9.3 $\pm$ 3.2#
37nM		121.8 $\pm$ 11.2		59.8 $\pm$ 16.9#		21.2 $\pm$ 10.3#
370nM		105.8 $\pm$ 7.9		30.0 $\pm$ 10.5		17.8 $\pm$ 9.1#
3700nM		82.2 $\pm$ 3.8*		6.3 $\pm$ 3.1#*		n.d
Oxytocin (day 15)						
2pM	100	96.1 $\pm$ 8.2	31.0 $\pm$ 3.6#	24.4 $\pm$ 3.8	10.9 $\pm$ 2.2#	6.7 $\pm$ 1.2#
20pM		108.4 $\pm$ 11.9		32.8 $\pm$ 7.3		12.1 $\pm$ 3.0#
200pM		98.0 $\pm$ 8.4		25.6 $\pm$ 3.9		8.6 $\pm$ 2.2#
Progesterone (day 15)						
3.2nM	100	104.9 $\pm$ 12.1	31.0 $\pm$ 3.6#	26.0 $\pm$ 4.1#	10.9 $\pm$ 2.2#	10.7 $\pm$ 2.9#
32nM		91.2 $\pm$ 8.3		24.5 $\pm$ 6.4#		10.7 $\pm$ 2.6#
320nM		88.8 $\pm$ 7.2		17.4 $\pm$ 3.2#*		7.3 $\pm$ 1.6#
3200nM		80.1 $\pm$ 7.5*		15.5 $\pm$ 3.3#*		6.9 $\pm$ 1.6#
Hydrocortisone (day 15)						
2.8nM	100	115.0 $\pm$ 13.1	33.7 $\pm$ 2.2	38.4 $\pm$ 6.2	11.1 $\pm$ 1.8	11.7 $\pm$ 2.0
28nM		128.7 $\pm$ 14.6		41.6 $\pm$ 4.8		16.0 $\pm$ 3.6
280nM		106.7 $\pm$ 14.6		37.1 $\pm$ 5.4		10.3 $\pm$ 2.0
2800nM		92.9 $\pm$ 5.8		29.6 $\pm$ 4.4		7.7 $\pm$ 1.6

\* significantly ( $P < 0.05$ ) lower than corresponding control (C) value on the same day of culture.

# Mean ( $\pm$  S.E.M.) of 4 to 8 values (other values n.d., i.e. below detection limit of 10pg/mg tissue /24h)



Table 6. Mean ( $\pm$  s.e.m.,  $n = 10$ ) output of 6-keto-PGF<sub>1 $\alpha$</sub>  (expressed as % of day 1 control (C) value) from day 7 and 15 (as indicated) cultured guinea-pig endometrium and not treated (C) or treated (T) with oestradiol, oxytocin, progesterone and hydrocortisone on all 3 days of culture.

Day of culture:		1			2			3		
Treatment:		C	T		C	T		C	T	
Output of 6-keto-PGF <sub>1<math>\alpha</math></sub> (% of control on day 1 of culture)										
Oestradiol (day 15)										
3.7nM	100	130.2 $\pm$ 17.6	33.3 $\pm$ 3.6	39.5 $\pm$ 4.7	19.5 $\pm$ 4.4	20.4 $\pm$ 4.4				
37nM		125.4 $\pm$ 16.0		34.4 $\pm$ 3.1		18.3 $\pm$ 2.3				
370nM		123.3 $\pm$ 13.9		36.5 $\pm$ 5.9		19.0 $\pm$ 4.3				
3700nM		99.3 $\pm$ 5.4		25.3 $\pm$ 2.2		14.6 $\pm$ 1.9				
Oestradiol (day 7)										
3.7nM	100	108.2 $\pm$ 7.8	24.9 $\pm$ 1.7	24.6 $\pm$ 1.8	13.4 $\pm$ 1.4	13.1 $\pm$ 1.5				
37nM		103.1 $\pm$ 5.3		26.1 $\pm$ 2.0		13.2 $\pm$ 1.7				
370nM		104.5 $\pm$ 9.0		23.9 $\pm$ 1.7		12.1 $\pm$ 1.5				
3700nM		100.7 $\pm$ 6.0		22.4 $\pm$ 1.9		9.8 $\pm$ 1.2				
Oxytocin (day 15)										
2pM	100	105.2 $\pm$ 10.1	24.8 $\pm$ 2.5	21.7 $\pm$ 2.1	9.5 $\pm$ 0.8	9.0 $\pm$ 0.8				
20pM		92.1 $\pm$ 6.0		22.6 $\pm$ 2.6		10.4 $\pm$ 1.1				
200pM		92.9 $\pm$ 6.7		24.4 $\pm$ 2.4		9.2 $\pm$ 0.7				
Progesterone (day 15)										
3.2nM	100	111.2 $\pm$ 8.2	24.8 $\pm$ 2.5	25.5 $\pm$ 2.1	9.5 $\pm$ 0.8	9.3 $\pm$ 0.8				
32nM		86.1 $\pm$ 5.5		21.5 $\pm$ 1.7		7.8 $\pm$ 0.8				
320nM		95.2 $\pm$ 7.9		21.9 $\pm$ 1.9		8.6 $\pm$ 0.8				
3200nM		96.5 $\pm$ 8.0		21.6 $\pm$ 2.0		8.4 $\pm$ 0.7				
Hydrocortisone (day 15)										
2.8nM	100	117.5 $\pm$ 8.3	31.9 $\pm$ 2.3	37.7 $\pm$ 6.6	10.2 $\pm$ 1.9	10.6 $\pm$ 1.6				
28nM		122.2 $\pm$ 10.7		33.7 $\pm$ 6.1		11.8 $\pm$ 1.9				
280nM		126.0 $\pm$ 10.1		28.8 $\pm$ 2.8		10.0 $\pm$ 1.2				
2800nM		104.9 $\pm$ 6.4		27.1 $\pm$ 1.8		11.0 $\pm$ 1.9				

## DISCUSSION

The amounts of PGs in the guinea-pig uterus are very low (23), so the PG content of the culture medium reflects fresh PG synthesis by the endometrium. The basal output of  $\text{PGF}_{2\alpha}$  from guinea-pig endometrium maintained in culture was higher on day 15 than on day 7 of the oestrous cycle. This shows that the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis and release, due to oestradiol acting on a progesterone-primed uterus from day 11 of the oestrous cycle in vivo (see Introduction), is maintained in tissue culture in the absence of steroid hormones, particularly during the first day of culture.  $\text{PGF}_{2\alpha}$  output falls during the next two days of culture, especially output from day 15 endometrium. From the report of Leaver & Seawright (11), it was expected that oestradiol (37 to 3700nM) added to the culture medium on day 2 of culture would have increased  $\text{PGF}_{2\alpha}$  output from day 7 and day 15 guinea-pig endometrium on day 3 of culture. Similarly, oxytocin treatment on day 2 of culture would have been expected to increase  $\text{PGF}_{2\alpha}$  output from day 15 guinea-pig endometrium on days 2 and 3 of culture. We found that oestradiol and oxytocin had no such stimulatory activity. Even when present for all 3 days of culture, oestradiol and oxytocin had no stimulatory effect on the output of  $\text{PGF}_{2\alpha}$ . The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were also not stimulated by oestradiol and oxytocin in both experiments. In fact, oestradiol at the highest concentration used (3700nM) reduced the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and, to a lesser extent, 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and 15 guinea-pig endometrium.

Our findings do not support the conclusions of Leaver & Seawright (11) that the in vitro treatment with oestradiol and oxytocin stimulates  $\text{PGF}_{2\alpha}$  output from guinea-pig endometrium maintained in tissue culture. This lack of effect of oxytocin on PG output is in agreement with the study of Poyser & Brydon (24) in which the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 7 and day 15 guinea-pig uterus were not affected during a 30 min superfusion with oxytocin. The failure of oxytocin in the present study to stimulate  $\text{PGF}_{2\alpha}$  release from the guinea-pig uterus is consistent with the failure of oxytocin treatment of guinea-pigs in vivo to shorten oestrous cycle length (25).

The lack of a stimulatory effect of oestradiol in vitro on  $\text{PGF}_{2\alpha}$  output from the guinea-pig endometrium maintained in tissue culture is in contrast to the well-documented increase in  $\text{PGF}_{2\alpha}$  output from human endometrium, maintained in tissue or cell culture, by oestradiol treatment in vitro (10,12,15,26). Although the treatment of guinea-pig endometrium in vitro with oestradiol does not stimulate  $\text{PGF}_{2\alpha}$  output, the in vivo treatment of guinea-pigs with physiological doses of oestradiol leads to an increase in uterine  $\text{PGF}_{2\alpha}$  output when measured in vivo or in vitro (4,7,27). However, the treatment of guinea-pigs with large, non-physiological doses of oestradiol prevents luteolysis due, in part, to an anti-

luteolytic action (28). The findings in the present study that a high concentration of oestradiol reduces  $\text{PGF}_{2\alpha}$  output from guinea-pig endometrium in culture provides a possible explanation for this anti-luteolytic effect.

Since oestradiol and oxytocin failed to stimulate PG output from guinea-pig endometrium in culture, it was not possible to investigate the effects of progesterone and hydrocortisone on hormonally-stimulated increases in endometrial  $\text{PGF}_{2\alpha}$  synthesis and release. However, progesterone inhibited the basal outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  but not of 6-keto- $\text{PGF}_{1\alpha}$ . This inhibitory effect of progesterone on  $\text{PGF}_{2\alpha}$  output is in agreement with similar studies performed on the guinea-pig (11) and human uterus (9,10,26). Studies on human endometrium indicated that progesterone inhibits PG synthesis by preventing the release of arachidonic acid from phospholipids due to it having an indirect inhibitory action on phospholipase activity (29). Since glucocorticoids inhibit phospholipase  $\text{A}_2$  activity (and, hence, arachidonic acid release) indirectly by causing the synthesis of lipocortin (see 13) and since glucocorticoids also inhibit  $\text{PGF}_{2\alpha}$  output from the human endometrium (15,25,29), progesterone may inhibit endometrial  $\text{PGF}_{2\alpha}$  synthesis and release due to it cross-reacting with the glucocorticoid receptor and causing the synthesis of lipocortin. However, in the present study hydrocortisone, over a wide concentration range, had no effect on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from guinea-pig endometrium in culture. In a previous study (30), hydrocortisone had no inhibitory effect on the basal and  $\text{A23187}$ -stimulated outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 15 guinea-pig uterus superfused for 3h *in vitro*. Consequently, hydrocortisone has no effect on  $\text{PGF}_{2\alpha}$  synthesis by the guinea-pig endometrium during short-term or long-term treatment with the hormone. Therefore, it is unlikely that the inhibitory effect of progesterone on endometrial  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs is due to a glucocorticoid-like action. This conclusion is in agreement with the study of Schatz, Markiewicz & Gursipide (31) who found that, while dexamethasone and progesterone both reduce  $\text{PGF}_{2\alpha}$  from human endometrium maintained in tissue culture, dexamethasone increased while progesterone decreased lipocortin output, indicating that progesterone and the glucocorticoid hormones inhibit  $\text{PGF}_{2\alpha}$  output from human endometrium by different mechanisms.

Progesterone inhibited the outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  from day 7 and 15 guinea-pig endometrium without affecting the output of 6-keto- $\text{PGF}_{1\alpha}$ . This finding indicates that the intracellular processes controlling  $\text{PGI}_2$  synthesis (as reflected by measuring 6-keto- $\text{PGF}_{1\alpha}$  output) are different from those controlling the synthesis of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ . It will be interesting to see if progesterone has a differential effect on endometrial PG synthesis in other species. The inhibitory effect of a large concentration of oestradiol may be due to it having a progesterone-like action. However oestradiol, in contrast to progesterone, tended to reduce

endometrial 6-keto-PGF<sub>1α</sub> output. Also, oestradiol (37μM) but not progesterone (32μM) inhibited the A23197-induced and arachidonic acid-induced increases in outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the day 7 and day 15 guinea-pig uterus superfused in vitro (30). All these findings suggest that the inhibitory actions of oestradiol and progesterone are different.

Overall, these studies have failed to confirm a previous report (11) that oestradiol and oxytocin treatment in vitro stimulates PGF<sub>2α</sub> output from guinea-pig endometrium maintained in tissue culture. The inhibitory effect of progesterone on endometrial PGF<sub>2α</sub> and PGE<sub>2</sub> outputs appears not to be due to a glucocorticoid-like action.

## ACKNOWLEDGEMENTS

This study was supported by a grant from the SERC, and an MRC Research Scholarship to S.C.R. The technical assistance of Miss L. Marshall is much appreciated. Authentic prostaglandins were kindly supplied by the Upjohn Co., Kalamazoo, Michigan, U.S.A.

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Editor: H. Behrman

Received: 5-26-87

Accepted: 8-26-87

# PROSTAGLANDINS LEUKOTRIENES AND MEDICINE

Prostaglandins Leukotrienes and Medicine (1987) 28, 153-168  
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## EFFECT OF ACTINOMYCIN D ON PROSTAGLANDIN SYNTHESIS BY AND OUTPUT FROM THE GUINEA-PIG UTERUS

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### ABSTRACT

The intra-uterine administration of actinomycin D on Day 10 reduced the output of prostaglandin (PG)  $F_{2\alpha}$  (the major PG released) from the Day 15 guinea-pig uterus *in vitro* by 80 to 85%.  $PGE_2$  output was reduced by 50%, while 6-keto- $PGF_{1\alpha}$  output was unaffected. Plasma progesterone levels were high (3 to 15 ng/ml) on Day 15 due to the reduction in uterine  $PGF_{2\alpha}$  output. Endometrial  $PGF_{2\alpha}$  synthesizing capacity was reduced by 50% by actinomycin D treatment, while endometrial  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  synthesizing capacities were unaffected. Oestradiol treatment *in vivo* did not reverse the inhibitory effects of actinomycin D on uterine PG production. A23187 increased uterine  $PGF_{2\alpha}$ , 6-keto- $PGF_{1\alpha}$  and  $PGE_2$  outputs irrespective of treatment, indicating that substrate supply was always rate limiting. Actinomycin D inhibited the uterotrophic action of oestradiol indicating that fresh protein synthesis had been inhibited. Overall, this study suggests that increased protein synthesis is involved in stimulating endometrial  $PGF_{2\alpha}$  synthesis and release. Previous studies have shown that increases in enzyme activities induced by oestradiol are only secondary events in the stimulation of endometrial  $PGF_{2\alpha}$  production. We propose that oestradiol induces the synthesis of a protein ('lipostimulin') which, acting on a progesterone-primed uterus, 'switches on' endometrial  $PGF_{2\alpha}$  synthesis and release by causing the activation of endometrial phospholipase  $A_2$ .



## INTRODUCTION

The life-span of the corpus luteum, and hence oestrous cycle length, in the guinea-pig, as in several other non-primate mammalian species, is controlled by prostaglandin (PG)  $F_{2\alpha}$  released from the uterus (1,2). Oestradiol acting on a progesterone-primed uterus is probably the physiological stimulus in guinea-pigs for the increase in  $PGF_{2\alpha}$  production by the uterus during the last one-third of the cycle (3,4). Oestradiol output from the ovary (5) increases just before the increase in  $PGF_{2\alpha}$  output from the uterus (6, 7, 8). It is generally recognised that many actions of oestradiol are mediated by increased protein synthesis which in turn is dependent upon the stimulation of DNA-dependent RNA synthesis. A previous study (9) showed that actinomycin D, an inhibitor of DNA-dependent RNA synthesis, administered directly into the uterine lumen prevented the increase in uterine  $PGF_{2\alpha}$  synthesizing capacity which normally occurs towards the end of the oestrous cycle, and prolonged the life-span of the corpus luteum as indicated by plasma progesterone levels and oestrous cycle length. It was presumed in that study that actinomycin D had prevented the increase in  $PGF_{2\alpha}$  output from the uterus, thus resulting in luteal maintenance. Consequently, in the present study, PG output from and endometrial PG synthesizing capacity by the guinea-pig uterus have been measured following the intra-uterine administration of actinomycin D and following A23187 treatment *in vitro*, so as to gain further insight into the intracellular processes controlling endometrial  $PGF_{2\alpha}$  synthesis and release.

## METHODS

Virgin, female guinea-pigs weighing 650-950 g were examined daily and a vaginal smear was taken when the vagina was perforate. Day 1 of the cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. All guinea-pigs used had exhibited cycles of normal length before being used in the following experiments. After receiving the appropriate treatment, each guinea-pig was killed on Day 15 of the cycle by stunning and incising the neck. A blood sample was collected into a heparinized (20 U/ml) syringe, and was centrifuged at 2500 g. The plasma was withdrawn and stored at - 20°C before being assayed for progesterone.

Experiment 1. The uteri from 5 Day-15 guinea-pigs were removed and divided into their 2 uterine horns. Each uterine horn was "opened" by cutting longitudinally. The endometrium and myometrium forming one uterine horn from each animal were separated by cutting pieces of endometrium away from the myometrium with a pair of fine scissors (10). Each tissue type was weighed, homogenized separately in 10 ml Krebs solution (for composition, see 11), and incubated for 60 min at 37°C in an atmosphere of 5%  $CO_2$  and 95%  $O_2$ .

The other uterine horn from each animal was weighed, suspended in an organ bath and attached to an isotonic lever under a load of 2 g. This uterine horn was superfused (5 ml/min) with Krebs solution at 37°C and which had been pre-gassed with 5%  $CO_2$  and 95%  $O_2$ . Samples of superfusate were collected for 10-min periods consecutively over the

first 70 min of superfusion (i.e. 7 samples). Immediately after the superfusion, the endometrium and myometrium were separated, weighed, homogenized and incubated as above. The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  in the incubates and superfusates were measured by radioimmunoassay (RIA).

Experiment 2. Ten Day-10 guinea-pigs were anaesthetized i.p. with fentanyl citrate (0.47 mg/Kg) and fluanisone (15 mg/Kg; "Hypnorm", Crown Chemical Co. Ltd., Lamberhurst, Kent), plus midazolam (3 mg/Kg; "Hypnovel", Roche Products Ltd., Welwyn Garden City). An incision was made in the abdomen of each guinea-pig, the junction of the 2 uterine horns was exposed, and 0.5 ml of sterile NaCl solution (9 g/l; "Steriflex", Boots Company Ltd., Nottingham) was injected into each uterine horn. For 5 of the guinea-pigs the NaCl solution contained 20  $\mu\text{g}$  of actinomycin D (Sigma Chemical Co. Ltd., Poole, Dorset). The body wall and skin were stitched and each guinea-pig received s.c. 0.4 mg naloxone ("Narcan", Du Pont (UK) Ltd., Stevenage, Herts.) to quicken recovery from the anaesthetic. None of the animals subsequently showed any adverse effects.

The uterus from each guinea-pig was removed on Day 15. The two uterine horns were separated, "opened" by cutting longitudinally and treated as in Exp. 1, except that only 2 samples of superfusate were collected. These samples were obtained between 0-10 min and 60-70 min, 0 min being the start of the period of superfusion. The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present in the superfusates and incubates were measured by RIA.

This whole procedure was repeated on a further 10 guinea-pigs with, additionally, each animal receiving s.c. 10  $\mu\text{g}$  oestradiol benzoate (Sigma Chemical Co. Ltd., Poole, Dorset) in 0.5 ml arachis oil daily between Days 11 and 14.

Experiment 3. Ten Day-10 guinea-pigs were anaesthetized and received intra-uterine NaCl solution (9 g/l) with or without actinomycin D (5 animals of each), as in Exp. 2. The uterine horns were removed on Day 15, weighed, "opened" by cutting longitudinally, and both horns superfused with Krebs solution using the same conditions as in Exp. 1. After a settling period of 60 min, samples of superfusate were collected from each uterine horn for 10-min periods consecutively over the next 90 min (i.e. 9 samples/uterine horn). During the superfusion of one uterine horn from each animal, A23187 (Sigma Chemical Co. Ltd., Poole, Dorset) was present in the Krebs solution (1  $\mu\text{g}/\text{ml}$ ) during the collection of samples 4, 5 and 6. Immediately after the end of the superfusion period, the endometrium and myometrium forming each uterine horn were separated, weighed, homogenized and incubated as in Exp. 1. The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  present in the superfusates and incubates were measured by RIA.

This whole procedure was repeated on a further 10 guinea-pigs with, additionally, each guinea-pig receiving s.c. 10  $\mu\text{g}$  oestradiol benzoate in 0.5 ml arachis oil daily between Days 11 and 14.

Table 1. Mean ( $\pm$  s.e.m.,  $n = 5$ ) amounts (ng/100 mg tissue) of prostaglandins (PGs) synthesized by homogenates of endometrium and myometrium from Day-15 guinea-pigs.

Time	Endometrium		Myometrium	
	0 min	70 min	0 min	70 min
PGF <sub>2</sub> $\alpha$	82.1 $\pm$ 8.7	55.1 $\pm$ 8.0 *	17.4 $\pm$ 1.2	19.5 $\pm$ 8.7
PGF <sub>2</sub>	13.4 $\pm$ 0.9	8.3 $\pm$ 0.1 *	8.0 $\pm$ 1.3	8.7 $\pm$ 0.3
6-keto-PGF <sub>1</sub> $\alpha$	31.1 $\pm$ 9.1	24.4 $\pm$ 1.7	58.7 $\pm$ 11.8	56.0 $\pm$ 5.1

\* significantly lower ( $P < 0.05$ ) than corresponding value at 0 min for same tissue and same PG.

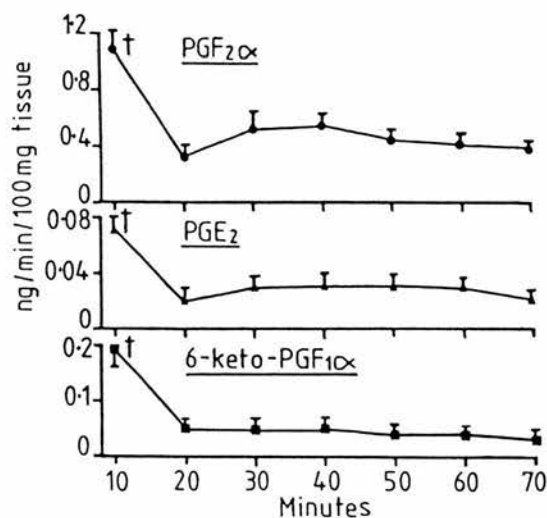


Figure 1. Mean ( $\pm$  s.e.m.,  $n = 5$ ) outputs of prostaglandins (PGs) from Day-15 guinea-pig uterine horns superfused in vitro.

† significantly higher ( $P < 0.05$ ) than the subsequent values for the same PG.

### Assay procedures

**Solvent extraction.** The pH of the superfusates and incubates was adjusted to 4.0 with HCl. Each sample was extracted twice by shaking with 2 volumes of ethyl acetate. The two ethyl acetate fractions were combined and evaporated to dryness at 45°C on a rotary evaporator. Each dried extract was redissolved in 5 ml (incubates) and 10 ml (superfusates) ethyl acetate and stored at -20°C before being assayed. The recoveries of PGF<sub>2α</sub> and PGE<sub>2</sub> are > 90%, and the recovery of 6-keto-PGF<sub>1α</sub> is > 80% by this method (12, 13). The results are not corrected for recoveries.

**Radioimmunoassays.** PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were measured using antibodies raised in rabbits and tested in this laboratory (12, 14, 15, 16). The only significant cross-reactivities are PGF<sub>1α</sub> (100%) with the PGF<sub>2α</sub> antiserum, and PGE<sub>1</sub> (94%), PGB<sub>2</sub> (73%) and PGA<sub>2</sub> (24%) with the PGE<sub>2</sub> antiserum. Previous studies involving analysis by gas chromatography and mass spectrometry have shown that the guinea-pig uterus synthesizes very little PGF<sub>1α</sub> in comparison to PGF<sub>2α</sub>, and that there is no detectable synthesis of PGE<sub>1</sub>, PGB<sub>2</sub> or PGA<sub>2</sub> (17). Consequently, the PGF<sub>2α</sub> and PGE<sub>2</sub> antisera were measuring predominantly the PG to which they had been raised. The intra-assay coefficients of variation, calculated from the variation between the duplicates results obtained for each sample were 5.6% (PGF<sub>2α</sub>), 7.8% (PGE<sub>2</sub>) and 5.9% (6-keto-PGF<sub>1α</sub>). The inter-assay coefficients of variation, calculated from the results obtained by incorporating a known amount of the appropriate PG into each assay, were 9.3% (PGF<sub>2α</sub>), 8.6% (PGE<sub>2</sub>) and 6.6% (6-keto-PGF<sub>1α</sub>). The limits of detection were 30 pg PGF<sub>2α</sub>, 40 pg PGE<sub>2</sub> and 30 pg 6-keto-PGF<sub>1α</sub> per assay tube for the respective assays.

Progesterone was measured as described previously (17), using an antibody raised in this laboratory and whose cross-reactivities have been reported elsewhere (18, 19). The intra-assay and inter-assay coefficients of variation (calculated as for the PGs) were 10.8% and 10.1%, respectively. The detection limit was 30 pg progesterone per assay tube.

**Statistical Tests.** Changes in the output of a PG with time during the superfusion experiments were analyzed by Duncan's multiple-range test. Other comparisons were made by using Student's *t* test (which was used in a modified form if the variances were unequal by the variance *F* ratio test), or by using the paired *t* test, as appropriate.

### RESULTS

Results are expressed as the mean ( $\pm$  s.e.m., *n* = 5 unless otherwise stated).

**Experiment 1.** The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the Day 15 superfused uterine horns were initially high during the first 10-min period of superfusion, but then significantly fell (*P* < 0.05) to values which were 50 - 80% lower than the initial values. The outputs of the 3 PGs did not vary significantly with time over the next 60-min period of superfusion (Fig. 1). The amounts of PGF<sub>2α</sub> and PGE<sub>2</sub>, but not of

Table 2. Mean ( $\pm$  s.e.m.,  $n = 5$ ) amounts of prostaglandins (PGs; ng/min/10 mg tissue), plasma progesterone levels (ng/ml) and uterine horn weights (Ut.Hn.Wts.; mg) released from Day-15 guinea-pigs receiving intra-uterine NaCl solution (9 g/l) on Day 10 not containing (control) or containing (Actin. D) 20  $\mu$ g actinomycin D, and not treated or treated with 10  $\mu$ g oestradiol benzoate s.c. daily from Days 11-14.

PG outputs				Progesterone	Ut.Hn.Wts.
PGF <sub>2</sub> α	PGE <sub>2</sub>	6-keto-PGF <sub>1</sub> α			
WITHOUT OESTRADIOL TREATMENT					
10 min:-					
Control	1.13±0.31	0.089±0.019	0.091±0.020	0.54±0.02	552±33
Actin.D.	0.19±0.01*	0.041±0.004*	0.142±0.031	8.54±1.99	620±63
70 min:-					
Control	0.25±0.04#	0.022±0.004#	0.012±0.002#	-	537±26
Actin.D.	0.05±0.01**	0.011±0.002**	0.012±0.004#	-	613±43
WITH OESTRADIOL TREATMENT					
10 min:-					
Control	1.00±0.18	0.185±0.059	0.139±0.023	0.48±0.13	858±56
Actin.D.	0.36±0.10*	0.087±0.015	0.106±0.013	6.93±0.82	725±31*
70 min:-					
Control	0.39±0.18#	0.056±0.005#	0.030±0.003#	-	856±44
Actin.D.	0.15±0.03**	0.018±0.003**	0.025±0.006#	-	705±32*

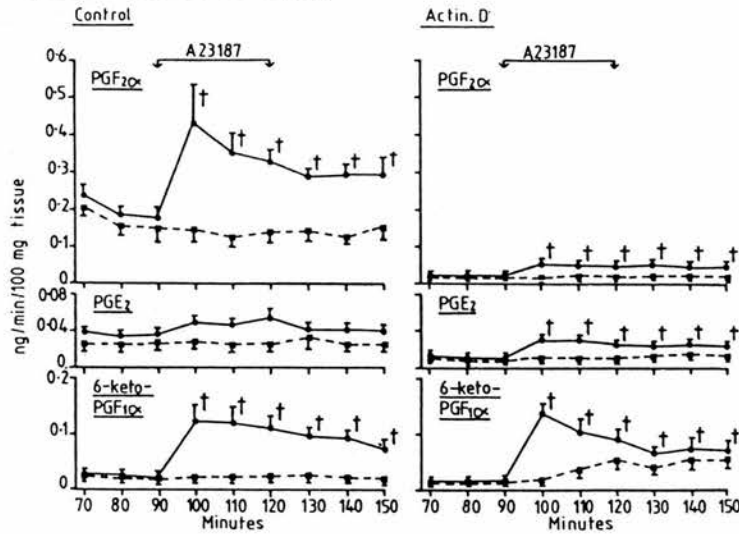
# significantly lower ( $P < 0.05$ ) than corresponding output at 10 min for same PG from uterine horns receiving the same treatment.  
 \* significantly lower ( $P < 0.05$ ) than corresponding "Control" value for same PG at the same time.

Table 3. Mean ( $\pm$  s.e.m.,  $n = 5$ ) amounts of prostaglandins (PGs; ng/100 mg tissue) synthesized by homogenates of endometrium and myometrium from Day-15 guinea-pigs receiving intra-uterine NaCl solution (9 g/l) on Day 10 not containing (Control) or containing (Actin. D) 20  $\mu$ g actinomycin D, and not treated or treated with 10  $\mu$ g oestradiol benzoate s.c. daily from Days 11-14.

Time	Endometrium			Myometrium		
	PGE <sub>2</sub> $\alpha$	PGE <sub>2</sub>	6-keto-PGF <sub>1</sub> $\alpha$	PGF <sub>2</sub> $\alpha$	PGE <sub>2</sub>	6-keto-PGF <sub>1</sub> $\alpha$
WITHOUT OESTRADIOL TREATMENT						
0 min:-						
Control	105.9 $\pm$ 14.1	16.8 $\pm$ 1.6	40.1 $\pm$ 5.4	26.8 $\pm$ 4.8	13.6 $\pm$ 1.8	65.1 $\pm$ 9.5
Actin.D.	46.8 $\pm$ 6.5 *	15.2 $\pm$ 1.2	31.9 $\pm$ 4.2	18.4 $\pm$ 2.1	11.7 $\pm$ 1.5	65.3 $\pm$ 11.8
70 min:-						
Control	64.2 $\pm$ 6.9 #	10.6 $\pm$ 0.5 #	19.7 $\pm$ 3.6 #	20.7 $\pm$ 1.3	10.6 $\pm$ 0.5	46.8 $\pm$ 7.0
Actin.D.	33.9 $\pm$ 8.1 *	12.6 $\pm$ 3.1	25.1 $\pm$ 4.8	17.4 $\pm$ 2.7	14.7 $\pm$ 1.9	62.4 $\pm$ 11.0
WITH OESTRADIOL TREATMENT						
0 min:-						
Control	70.7 $\pm$ 13.4	22.1 $\pm$ 3.5	22.9 $\pm$ 5.7	13.9 $\pm$ 2.9	6.8 $\pm$ 0.9	19.7 $\pm$ 4.6
Actin.D.	22.6 $\pm$ 2.1 *	9.6 $\pm$ 1.3 *	6.5 $\pm$ 0.5 *	12.6 $\pm$ 1.2	10.7 $\pm$ 1.3	14.7 $\pm$ 2.4
70 min:-						
Control	68.6 $\pm$ 12.3	26.1 $\pm$ 4.6	25.3 $\pm$ 5.6	11.9 $\pm$ 3.0	7.4 $\pm$ 0.8	21.7 $\pm$ 4.9
Actin.D.	24.4 $\pm$ 3.5 *	11.8 $\pm$ 1.1 *	8.4 $\pm$ 0.8 *	9.9 $\pm$ 0.5	8.6 $\pm$ 1.4	13.8 $\pm$ 0.9

# significantly lower ( $\bar{P} < 0.05$ ) than corresponding value at 0 min for same PG from same tissue receiving same treatment.  
 \* significantly lower ( $\bar{P} < 0.05$ ) than corresponding "Control" value for same PG at the same time and for the same tissue.

# 1. Without Oestradiol Treatment



# 2. With Oestradiol Treatment

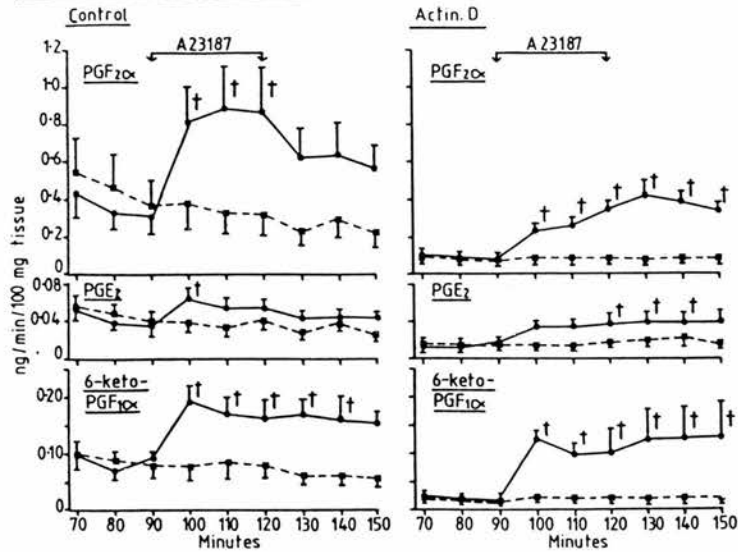


Figure 2. Mean ( $\pm$  s.e.m.,  $n = 5$ ) outputs of prostaglandins (PGs) from the uterine horns superfused in vitro of Day-15 guinea-pigs receiving intra-uterine NaCl solution (9 g/l) on Day 10 not containing (Control) or containing (Actin.D.) 20  $\mu$ g actinomycin D, (1) not treated or (2) treated with 10  $\mu$ g oestradiol benzoate s.c. daily from Days 11-14, and not treated (broken line) or treated (solid line) with A23187 (1  $\mu$ g/ml) as indicated.

† significantly higher ( $P < 0.05$ ) than corresponding values for the same PG before treatment with A23187.



6-keto-PGF<sub>1α</sub>, synthesized by the endometrium significantly fell ( $P < 0.05$ ) between 0 and 70 min. The amounts of the 3 PGs synthesized by the myometrium did not differ significantly between the 2 times studied (Table 1). Plasma progesterone levels in this group of Day-15 guinea-pigs were  $0.27 \pm 0.05$  ng/ml.

Experiment 2. The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from all uterine horns, with or without actinomycin D treatment, were significantly lower ( $P < 0.05$ ) at 70 min than at 10 min (Table 2). The outputs of PGF<sub>2α</sub> and of PGE<sub>2</sub>, but not of 6-keto-PGF<sub>1α</sub>, were significantly lower from the actinomycin D-treated uterine horns than from the control uterine horns, at both times studied (Table 2). A similar profile of PG outputs was found in those guinea-pigs which had additionally received oestradiol benzoate between Days 11 and 14, except that PGE<sub>2</sub> output from the actinomycin D-treated uterine horns was only significantly ( $P < 0.05$ ) lower at 70 min (Table 2).

The amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesized by the endometrium of control, but not of actinomycin D-treated, uterine horns significantly decreased ( $P < 0.05$ ) between 0 and 70 min in those guinea-pigs which did not receive oestradiol benzoate, but did not change significantly in those control guinea-pigs which received oestradiol benzoate between Days 11 and 14. The amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesized by the myometrium did not change with time in either group of guinea-pigs (Table 3).

In guinea-pigs which did not receive exogenous oestradiol, intra-uterine actinomycin D significantly ( $P < 0.05$ ) reduced the amounts of PGF<sub>2α</sub>, but not of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>, synthesized by the endometrium whereas in those guinea-pigs which received oestradiol, the amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesized by the endometrium were all significantly ( $P < 0.05$ ) reduced by actinomycin D treatment, at both times studied (Table 3). Actinomycin D did not affect PG synthesis by the myometrium in either group at either time (Table 3).

Plasma progesterone levels were high in those guinea-pigs which had received intra-uterine actinomycin D, with or without oestradiol treatment, and were typically low in the control Day-15 guinea-pigs (Table 2). The increase in uterine horn weights induced by oestradiol was significantly reduced ( $P < 0.05$ ) by actinomycin D (Table 2).

Experiment 3. A23187 caused a significant ( $P < 0.05$ ) stimulation in the outputs of PGF<sub>2α</sub>, 6-keto-PGF<sub>1α</sub> and, to lesser extent, PGE<sub>2</sub> from the guinea-pig uterus irrespective of the treatment they had received *in vivo* (Fig. 2). The maximum output of PGF<sub>2α</sub> was significantly lower ( $P < 0.05$ ) from the actinomycin D-treated uterine horns, with or without oestradiol treatment (Fig. 2). However, if the difference in the basal outputs of PGF<sub>2α</sub> are taken into account, A23187 caused an approximate 3-fold stimulation of uterine PGF<sub>2α</sub> output from control and actinomycin D-treated uterine horns, with or without oestradiol treatment.

A23187 treatment had no significant effect on the amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesized by homogenates of the endometrium or myometrium at the end of the superfusion period. In guinea-pigs which



Table 4. Mean ( $\pm$  s.e.m.,  $n = 5$ ) amounts of prostaglandins (PGs; ng/100mg tissue) synthesized by homogenates of endometrium and myometrium from Day-15 guinea-pigs receiving intra-uterine NaCl solution (9 g/l) on Day 10 not containing (Control) or containing (Actin.D.) 20  $\mu$ g actinomycin D, not treated or treated with 10  $\mu$ g oestradiol benzoate s.c. daily from Days 11-14, and following 150 min superfusion of the uterine horns in vitro not treated (-) or treated (+) with A23187.

Endometrium				Myometrium			
-A23187				+A23187			
Control	Actin.D.	Control	Actin.D.	Control	Actin.D.	Control	Actin.D.
WITHOUT OESTRADIOL TREATMENT							
PGF <sub>2</sub> $\alpha$	37.2 $\pm$ 10.0	5.3 $\pm$ 1.4*	22.4 $\pm$ 5.8	5.3 $\pm$ 1.9*	15.1 $\pm$ 3.2	5.2 $\pm$ 1.8*	12.4 $\pm$ 2.3
PGE <sub>2</sub>	8.9 $\pm$ 1.6	6.4 $\pm$ 0.8	7.3 $\pm$ 0.8	6.2 $\pm$ 2.0	10.0 $\pm$ 0.7	7.0 $\pm$ 1.2	7.9 $\pm$ 1.2
6-keto							
-PGF <sub>1</sub> $\alpha$	25.6 $\pm$ 3.4	18.1 $\pm$ 1.4	18.1 $\pm$ 1.4	18.9 $\pm$ 5.2	57.4 $\pm$ 8.4	42.5 $\pm$ 9.3	44.5 $\pm$ 7.6
							41.4 $\pm$ 6.0
WITH OESTRADIOL TREATMENT							
PGF <sub>2</sub> $\alpha$	68.6 $\pm$ 12.4	24.4 $\pm$ 3.5*	70.7 $\pm$ 13.4	22.6 $\pm$ 2.1*	14.9 $\pm$ 3.0	9.9 $\pm$ 0.5	13.9 $\pm$ 2.9
PGE <sub>2</sub>	26.1 $\pm$ 4.6	11.8 $\pm$ 1.1*	22.1 $\pm$ 3.5	9.6 $\pm$ 1.3*	7.4 $\pm$ 0.8	8.6 $\pm$ 1.5	6.8 $\pm$ 0.9
6-keto							
-PGF <sub>1</sub> $\alpha$	25.3 $\pm$ 5.6	8.4 $\pm$ 0.8*	22.9 $\pm$ 5.7	6.1 $\pm$ 0.5*	21.7 $\pm$ 4.9	13.8 $\pm$ 0.9	19.7 $\pm$ 4.6
							14.7 $\pm$ 2.4

\* significantly lower ( $P < 0.05$ ) than corresponding "Control" value for same PG from same tissue receiving similar treatment.

did not receive oestradiol treatment, the amounts of  $\text{PGF}_{2\alpha}$ , but not of  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$ , synthesized by the endometrium and myometrium were significantly lower ( $P < 0.05$ ) in the actinomycin D-treated than in control guinea-pigs (Table 4). Plasma progesterone levels were  $4.13 \pm 0.52$  and  $0.21 \pm 0.04$  ng/ml in the actinomycin D-treated and control guinea-pigs, respectively. In the oestradiol-treated guinea-pigs, the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesized by the endometrium, but not by the myometrium, were significantly lower ( $P < 0.05$ ) in the actinomycin D-treated than in the control guinea-pigs (Table 4). Plasma progesterone levels were  $3.31 \pm 0.80$  and  $0.11 \pm 0.02$  ng/ml in these 2 groups of guinea-pigs, respectively. The increase in uterine horn weight produced by oestradiol was significantly inhibited ( $P < 0.05$ ) by actinomycin D treatment ( $910 \pm 43$  v.  $596 \pm 24$  mg, respectively,  $n = 10$ ).

### DISCUSSION

The output of  $\text{PGF}_{2\alpha}$  from the superfused guinea-pig uterus *in vitro* increases 22-fold between Days 7 and 15 of the cycle (20). The release rates of  $\text{PGF}_{2\alpha}$  from the uterus observed *in vitro* are similar to those observed *in vivo* (21). The intra-uterine, but not systemic administration, of 40  $\mu\text{g}$  actinomycin D extends oestrous cycle length 2-fold, due to an extended life-span of the corpus luteum as indicated by plasma progesterone levels (9). The present study has shown that the intra-uterine administration of actinomycin D causes a 80-85% reduction in the output of  $\text{PGF}_{2\alpha}$  from the Day 15 guinea-pig uterus. Plasma progesterone levels were consequently high (for Day 15) in these guinea-pigs treated with actinomycin D. Oestradiol treatment did not overcome the inhibitory effect of intra-uterine actinomycin D on uterine  $\text{PGF}_{2\alpha}$  output and the resultant maintenance of luteal function.

The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (which reflects  $\text{PGI}_2$  production) from the superfused uterus of Day-15 guinea-pigs which did not receive actinomycin D were considerably lower than the output of  $\text{PGF}_{2\alpha}$ , as reported previously (20). Actinomycin D significantly reduced the output of  $\text{PGE}_2$ , but not of 6-keto- $\text{PGF}_{1\alpha}$ , irrespective of whether or not the guinea-pigs were treated with oestradiol. These findings show that actinomycin D is showing selectivity of action towards  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$ .

The  $\text{PGF}_{2\alpha}$  synthesizing capacity of the guinea-pig endometrium increases 2.5-fold between Days 7 and 15 of the cycle (18). Intra-uterine actinomycin D treatment prevented this increase in endometrial synthesizing capacity without affecting myometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity, and without affecting endometrial and myometrial  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacities. This shows that the inhibition of  $\text{PGF}_{2\alpha}$  synthesizing capacity by actinomycin D in homogenates of whole guinea-pig uterus (9) is due to a specific action of actinomycin D on the endometrium. Furthermore, this previous study (9) showed that the reduction of  $\text{PGF}_{2\alpha}$  synthesis in uterine homogenates by actinomycin D was not due to lack of available substrate or to increased  $\text{PGF}_{2\alpha}$  metabolism. Thus, the stimulation of endometrial PG synthetase levels (probably the cyclo-oxygenase component) by oestradiol (18) was prevented by the intra-uterine administration of actinomycin D, indicating that fresh

protein synthesis had been inhibited. Oestradiol treatment did not overcome this inhibitory effect of actinomycin D and, in fact, actinomycin D significantly reduced the endometrial, but not myometrial,  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesizing capacities in oestradiol-treated guinea-pigs.

It might be supposed that the reduced output of  $\text{PGF}_{2\alpha}$  from the guinea-pig uterus following actinomycin D treatment is due to the reduction in endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity. This would mean that the amount of enzyme and not the amount of free substrate is rate limiting in the synthesis of  $\text{PGF}_{2\alpha}$  by the endometrium. In control guinea-pigs which did not receive actinomycin D or oestradiol, endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity fell by 40% between 0 and 70 min (Table 3), and fell by another 50% between 70 and 150 min (cf. Tables 3 & 4). The endometrial synthesizing capacities of  $\text{PGE}_{1\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  also fell between 0 and 70 min, but did not decrease further between 70 and 150 min. However, after the initial high outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  during the first 10-min period of superfusion (probably due to the trauma of setting up the tissue), the basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  remained steady over the subsequent periods of superfusion (Figs 1 & 2), in spite of the reduction in endometrial PG synthesizing capacity. In addition, previous studies on ovariectomized guinea-pigs have shown that oestradiol causes the maximum increase in endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity, an effect which is reduced by progesterone (18). However, oestradiol acting on a progesterone-primed uterus causes a larger increase in  $\text{PGF}_{2\alpha}$  output from the uterus when compared to oestradiol acting alone (4). Also, although  $\text{PGF}_{2\alpha}$  is the major PG synthesized by homogenates of Day 7 guinea-pig endometrium, the output of  $\text{PGF}_{2\alpha}$  from the Day 7 superfused guinea-pig uterus is less than the outputs of  $\text{PGE}_2$ , 6-keto- $\text{PGF}_{1\alpha}$ , and thromboxane  $\text{B}_2$  (18, 20). These studies show that uterine  $\text{PGF}_{2\alpha}$  output is not controlled by endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity and that changes in endometrial  $\text{PGF}_{2\alpha}$  synthesizing capacity are not the direct cause of changes in uterine  $\text{PGF}_{2\alpha}$  output. Interestingly, oestradiol treatment prevented the decline in endometrial PG synthesizing capacity with time (Tables 3 & 4).

A23187 caused a 3-fold increase in uterine  $\text{PGF}_{2\alpha}$  output from all uterine horns, irrespective of treatment. The outputs of 6-keto- $\text{PGF}_{1\alpha}$  and, to a lesser extent,  $\text{PGE}_2$  were also increased. These increases are due to an influx of extracellular  $\text{Ca}^{2+}$  by A23187 (22) which presumably stimulates phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ; EC 3.1.1.4) to release more arachidonic acid for increased PG synthesis, since  $\text{PLA}_2$  is a calcium-requiring enzyme (23). Actinomycin D did not inhibit this process, initiated by A23187. The studies with A23187 indicate that the amount of available substrate and not the amount of enzyme is therefore the rate-limiting factor in controlling uterine  $\text{PGF}_{2\alpha}$  output (and the outputs of the other PGs), although the maximum amount of  $\text{PGF}_{2\alpha}$  synthesized would appear to depend to some extent on the amount of cyclo-oxygenase present in the endometrium.  $\text{PLA}_2$  activity in guinea-pig endometrium increases 1.5-fold between Days 7 and 16 of the cycle. However, optimum endometrial  $\text{PLA}_2$  activity is more than adequate to account for the amounts of  $\text{PGF}_{2\alpha}$  released from the uterus at both stages

of the cycle (23). It was considered, therefore, that activation of endometrial PLA<sub>2</sub> by raising the intracellular free Ca<sup>2+</sup> concentration, rather than changes in absolute PLA<sub>2</sub> activity, was the more important in the process by which oestradiol acting on a progesterone-primed uterus "switches on" endometrial PGF<sub>2α</sub> synthesis and release after Day 11 of the cycle in vivo.

In the present study, actinomycin D prevented this "switching on" process, and this inhibition was not overcome by oestradiol administration in a dose which normally stimulates uterine PGF<sub>2α</sub> synthesis and release (3, 4, 6). Actinomycin D also prevented fresh protein synthesis as indicated by the inhibition of the uterotrophic effect of oestradiol and the lack of increase in endometrial PG synthetase levels. Since many actions of oestradiol are mediated by increased protein synthesis, we propose, therefore, that oestradiol causes the synthesis by the endometrium of a protein ('lipostimulin') which acts in a progesterone-primed uterus to raise the endometrial intracellular free Ca<sup>2+</sup> concentration. This calcium combines with calmodulin (24, 25) which then activates PLA<sub>2</sub> (26) to release arachidonic acid from endometrial phospholipids, predominantly from phosphatidylcholine and phosphatidylethanolamine (10, 27), for increased PG synthesis. The increases in PLA<sub>2</sub> and cyclo-oxygenase activities are secondary events which ensure that, following stimulation of PLA<sub>2</sub> by lipostimulin, a greater amount of arachidonic acid is released and a greater proportion of that free arachidonic acid is converted into PGH<sub>2</sub> than would have occurred if the amounts of these enzymes present in the endometrium had not changed. The PGH<sub>2</sub> formed is predominantly directed in some way into the PGF<sub>2α</sub>-forming pathway. Whether such a lipostimulin exists requires further investigation.

Actinomycin D or cycloheximide administered to ovariectomized rats failed to prevent oestradiol treatment from stimulating uterine PG synthesis (28). From these findings, it was suggested (28) that oestrogen-stimulated uterine PG synthesis does not require protein synthesis in the uterus. This suggestion does not agree with our proposal. However, the protein synthesis inhibitors were administered to rats systemically and, in a previous study (9), it was shown that the systemic administration of actinomycin D to guinea-pigs had no effect on uterine PG synthesizing capacity and oestrous cycle length. It was found necessary to administer the actinomycin D directly into the guinea-pig uterus in order to prevent the stimulation of uterine PG production. Consequently, the study on rats needs repeating but with the protein synthesis inhibitors being administered by the intra-uterine route.

Actinomycin D and the anti-luteolytic factor secreted by the guinea-pig conceptuses (21) exert the same effects on the guinea-pig uterus after Day 11, i.e. prevent increases in uterine PGF<sub>2α</sub> output and endometrial PGF<sub>2α</sub> synthesizing capacity. Oestradiol treatment does not overcome these inhibitory effects in actinomycin D-treated guinea-pigs and in a majority (75%) of the pregnant guinea-pigs (19). Also, the uterotrophic action of oestradiol is prevented by actinomycin D and by the presence of the conceptuses. It appears that the anti-luteolytic factor, like actinomycin D, prevents oestradiol-induced fresh protein

synthesis from occurring in the endometrium, particularly the synthesis of the postulated lipostimulin. Whether the anti-luteolytic factor prevents oestradiol from combining with its receptor, or blocks the subsequent transcription or translation processes involved in protein synthesis, requires further study. In addition, it will also be interesting to see whether proteins secreted by the sheep and cow conceptus and which inhibit uterine PGF<sub>2α</sub> output induced by oestradiol (29, 30) also act by inhibiting the formation of uterine proteins required for the stimulation of endometrial PGF<sub>2α</sub> synthesis and release in these species. As in the guinea-pig, the intra-uterine administration of actinomycin D in sheep prolongs luteal function (31) indicating that fresh protein synthesis is normally required for the stimulation of uterine PGF<sub>2α</sub> production in the sheep.

#### ACKNOWLEDGEMENTS

This study was supported by a grant from the SERC, and by a MRC postgraduate studentship to S.C.R. The technical assistance of Miss Lorna Marshall is greatly appreciated. Authentic PGs were kindly supplied by the Upjohn Co., Kalamazoo, Michigan, U.S.A.

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# Prostaglandin production by the guinea-pig endometrium: is calcium necessary?

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RECEIVED 24 October 1986

## ABSTRACT

The output of prostaglandin (PG)  $F_{2\alpha}$  from guinea-pig endometrium obtained on day 15 of the oestrous cycle and maintained in tissue culture was significantly ( $P < 0.05$ ) reduced by the use of  $Ca^{2+}$ -depleted medium, EGTA (a  $Ca^{2+}$  chelator), 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8; an intracellular  $Ca^{2+}$  antagonist), trifluoperazine (TFP) and N-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W-7; both calmodulin antagonists). Nifedipine inhibited  $PGF_{2\alpha}$  output at a concentration (100  $\mu\text{mol/l}$ ) much greater than that usually required to block  $Ca^{2+}$  channels. Verapamil had a small but significant ( $P < 0.05$ ) inhibitory effect on  $PGF_{2\alpha}$  output at 10–100  $\mu\text{mol/l}$ . The outputs of  $PGE_2$  and, to a lesser extent, 6-keto- $PGF_{1\alpha}$  (the hydrated product of  $PGI_2$ ) were also reduced by using  $Ca^{2+}$ -depleted medium. EGTA reduced the outputs of  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  on day 1 of culture, but stimulated 6-keto- $PGF_{1\alpha}$  output on day 3 of culture.

The outputs of  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  were increased by TMB-8 (100  $\mu\text{mol/l}$ ) on day 3 of culture and by TFP and, to a smaller extent, by W-7 on all 3 days of culture. Nifedipine (100  $\mu\text{mol/l}$  by not 1 or 10  $\mu\text{mol/l}$ ) reduced the outputs of  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  on all 3 days of culture, whereas verapamil (100  $\mu\text{mol/l}$  but not 1 or 10  $\mu\text{mol/l}$ ) increased the outputs of these two prostaglandins on days 2 and 3 of culture. Phorbol 12-myristate 13-acetate (an activator of protein kinase C) had no effect on the outputs of  $PGF_{2\alpha}$ ,  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  from cultured guinea-pig endometrium obtained on days 7 and 15 of the oestrous cycle. It is concluded that extracellular  $Ca^{2+}$  is necessary for the high output of  $PGF_{2\alpha}$  from the guinea-pig uterus after day 11 of the oestrous cycle, and that the action of  $Ca^{2+}$  is not potentiated by the activation of protein kinase C.

*J. Endocr.* (1987) **113**, 463–471

## INTRODUCTION

The uterine luteolytic hormone in non-primate mammalian species has been identified as prostaglandin (PG)  $F_{2\alpha}$  (see Horton & Poyser, 1976; Poyser, 1981). In the guinea-pig, increased  $PGF_{2\alpha}$  output from the uterus occurs from day 11 of the cycle (Blatchley, Donovan, Horton & Poyser, 1972; Earthy, Bishop & Flack, 1975; Antonini, Turner & Pauerstein, 1976). Studies on the superfused guinea-pig uterus *in vitro* have shown that the outputs of  $PGF_{2\alpha}$ ,  $PGE_2$  and 6-keto- $PGF_{1\alpha}$  increase 21.9-, 1.8- and 2.9-fold respectively between days 7 and 15 of the cycle (Poyser & Brydon, 1983), and that oestradiol acting on a progesterone-primed uterus *in vivo* is responsible for this relatively specific increase in  $PGF_{2\alpha}$  output *in vitro* (Poyser, 1983a). Oestradiol output from the guinea-pig ovary increases after day 10 (Joshi, Watson & Labhsetwar, 1973), and this is consistent with oestradiol being responsible for the increase in uterine

$PGF_{2\alpha}$  output from day 11. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) appear to be the source of arachidonic acid for increased  $PGF_{2\alpha}$  synthesis by the guinea-pig endometrium (Leaver & Poyser, 1981; Ning, Leaver & Poyser, 1983; Ning & Poyser, 1984).

The calcium ionophore A23187 stimulates PG output from the guinea-pig uterus (Poyser & Brydon, 1983), an action dependent upon extracellular  $Ca^{2+}$  (Poyser, 1984). It has been proposed, therefore, that oestradiol acts upon a progesterone-primed uterus to increase the endometrial intracellular free  $Ca^{2+}$  concentration which, in turn, activates phospholipase (PL)  $A_2$  (EC 3.1.1.4; a calcium-requiring enzyme) to release arachidonic acid from PC and PE for  $PGF_{2\alpha}$  synthesis (Downing & Poyser, 1983). This study investigated whether  $Ca^{2+}$  is necessary for the high output of  $PGF_{2\alpha}$  from guinea-pig endometrium towards the end of the oestrous cycle.



## MATERIALS AND METHODS

Virgin female guinea-pigs, weighing between 600 and 950 g, were examined daily and a vaginal smear was taken when the vaginal membrane was open. The first day of the oestrous cycle was taken as the day before the post-ovulatory influx of leucocytes when cornification is at a maximum. All animals had normal cycles before being used. Each guinea-pig was killed by stunning and incising the neck on day 7 or day 15 of the cycle. The uterus was removed and placed in culture medium. The endometrium was dissected away from the myometrium under sterile conditions at room temperature by cutting away  $1 \times 2$  mm pieces of endometrium. Approximately 12 mg endometrium was placed on a raised platform in a Petri dish containing 4 ml Medium 199 plus Earle's salts or  $\text{Ca}^{2+}$ -depleted Medium 199 plus Earle's salts (the  $\text{Ca}^{2+}$ -containing salts having been omitted during preparation by the manufacturers), supplemented with glutamine and antibiotics (Ning *et al.* 1983). Eighteen Petri dishes containing endometrial tissue were prepared from the uterus of each guinea-pig; 14 of the dishes contained 'treatments' (performed in duplicate) as outlined below and four dishes were untreated (controls). The Petri dishes were equally divided between two Kilner jars (i.e. nine dishes per jar); each set of nine dishes consisted of one dish from each of the duplicate treatments and two untreated control dishes. The endometrium was cultured for 72 h as described previously, and the tissue remained viable during this period (Ning *et al.* 1983; Ning & Poyser, 1984). The culture medium was changed every 24 h. The samples of culture medium were stored at  $-20^\circ\text{C}$  before radioimmunoassay, without extraction, for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ .

### Experimental treatments

#### Using $\text{Ca}^{2+}$ -containing medium

Endometrium removed from guinea-pigs on day 15 of the oestrous cycle (day-15 guinea-pig endometrium) was treated with the following compounds: EGTA (a calcium chelator; 2 mmol/l), 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8; an intracellular  $\text{Ca}^{2+}$  antagonist; 30 and 100  $\mu\text{mol/l}$ ; Chiou & Malagodi, 1975), trifluoperazine (TFP; a calmodulin antagonist; 100 and 200  $\mu\text{mol/l}$ ; Levin & Weiss, 1977), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7; a calmodulin antagonist; 150 and 300  $\mu\text{mol/l}$ ; Hidaka, Asano, Iwadare *et al.* 1978), nifedipine (1, 10 and 100  $\mu\text{mol/l}$ ) and verapamil (1, 10, 50 and 100  $\mu\text{mol/l}$ ) (voltage-dependent  $\text{Ca}^{2+}$  channel blockers) and phorbol 12-myristate 13-acetate (TPA; a stimulator of protein kinase C; 1.6, 8.1 and 40.5 nmol/l; Castagna, Takai, Kaibuchi *et al.* 1982).

The preparation and dispensing of solutions containing nifedipine and verapamil were performed under sodium light.

Endometrium removed on day 7 of the oestrous cycle (day-7 guinea-pig endometrium) was cultured in medium containing TPA (1.6, 8.1 and 40.5 nmol/l).

#### Using $\text{Ca}^{2+}$ -depleted medium

Day-15 guinea-pig endometrium was cultured with EGTA (2 mmol/l), TMB-8 (100  $\mu\text{mol/l}$ ), W-7 (150  $\mu\text{mol/l}$ ), nifedipine (100  $\mu\text{mol/l}$ ) and verapamil (100  $\mu\text{mol/l}$ ).

### Sources of materials

Culture medium ( $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -depleted), glutamine and antibiotics were obtained from Flow Laboratories, Rickmansworth, Herts; Petri dishes from Sterilin Ltd, Teddington, Middx; EGTA and TPA from Sigma Chemical Co. Ltd, Poole, Dorset; TFP from Smith, Kline & French Laboratories, Welwyn Garden City, Herts; W-7 from Beecham-Wulfring, Gronau (Leine), F.R.G.; TMB-8 from Aldrich Chemical Co. Ltd, Gillingham, Kent; nifedipine from Bayer U.K. Ltd, Newbury, Berks and verapamil from Knoll AG, Ludwigshafen, F.R.G.

### Details of radioimmunoassay

Prostaglandin  $\text{F}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were measured using antibodies raised in this laboratory and whose cross-reactivities have been reported elsewhere (Dighe, Emslie, Henderson *et al.* 1975; Poyser, 1980; Poyser & Scott, 1980; Lytton & Poyser, 1982). The only significant cross-reactivities were  $\text{PGE}_1$  (66%),  $\text{PGA}_2$  (25.5%) and  $\text{PGB}_2$  (11.8%) with the  $\text{PGE}_2$  antiserum, and  $\text{PGF}_{1\alpha}$  (100%) with the  $\text{PGF}_{2\alpha}$  antiserum. Previous studies involving analysis by gas chromatography and mass spectrometry have shown that the guinea-pig uterus synthesizes very little  $\text{PGF}_{1\alpha}$  in comparison with  $\text{PGF}_{2\alpha}$ , and that there is no detectable synthesis of  $\text{PGE}_1$ ,  $\text{PGB}_2$ , and  $\text{PGA}_2$  (Poyser, 1983b). It is likely, therefore, that the  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  antibodies were measuring predominantly the PG to which they had been raised. The intra-assay coefficients of variation were 5.6% ( $\text{PGF}_{2\alpha}$ ), 7.8% ( $\text{PGE}_2$ ) and 5.9% (6-keto- $\text{PGF}_{1\alpha}$ ). The interassay coefficients of variation were 8.2% ( $\text{PGF}_{2\alpha}$ ), 8.6% ( $\text{PGE}_2$ ) and 6.6% (6-keto- $\text{PGF}_{1\alpha}$ ). The limits of detection were 0.09 pmol  $\text{PGF}_{2\alpha}$ , 0.11 pmol  $\text{PGE}_2$  and 0.08 pmol 6-keto- $\text{PGF}_{1\alpha}$  per assay tube for the respective assays.

### Statistical tests

Results were analysed by the one- or two-tailed Student's *t*-test, as appropriate.

## RESULTS

Using Ca<sup>2+</sup>-containing medium*Basal PG output from day-7 and day-15 guinea-pig endometrium in culture*

The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day-7 and day-15 guinea-pig endometrium declined from day 1 to day 3 of culture (Fig. 1). Comparing day-15 with day-7 tissue, the ratios of the mean outputs on days 1, 2 and 3 of culture for PGF<sub>2α</sub> were 7.2:1.0, 3.3:1.0 and 2.1:1.0 respectively, for PGE<sub>2</sub> they were 3.3:1.0, 2.4:1.0 and 1.3:1.0 respectively, and for 6-keto-PGF<sub>1α</sub> they were 1.2:1.0, 1.3:1.0 and 1.2:1.0 respectively. Output of PGF<sub>2α</sub> from day-15 endometrium was significantly ( $P < 0.05$ ) higher than from day-7 endometrium on all 3 days of culture. Output of PGE<sub>2</sub> was significantly ( $P < 0.05$ ) higher from day-15 endometrium on days 1 and 2 of culture, and 6-keto-PGF<sub>1α</sub> output was significantly ( $P < 0.05$ ) higher from day-15 endometrium only on day 2 of culture. The ratios of the mean outputs of PGF<sub>2α</sub>, 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> on days 1, 2 and 3 of culture for day-7 endometrium were 7.3:11.0:1.0, 9.8:4.8:1.0 and 6.7:4.3:1.0 respectively on the successive days of culture, and for day-15 endometrium they were 13.9:3.4:1.0, 13.4:2.6:1.0 and 8.2:3.2:1.0 respectively on the successive days of culture. PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub> were released in similar quantities from day-7 endometrium, with lesser quantities of PGE<sub>2</sub>. PGF<sub>2α</sub> was the major PG released from day-15 endometrium, with lesser quantities of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>.

*Effect of various treatments on PG output*

The results for day-15 guinea-pig endometrium are shown in Tables 1 (PGF<sub>2α</sub>), 2 (PGE<sub>2</sub>) and 3 (6-keto-PGF<sub>1α</sub>). EGTA (2 mmol/l) significantly ( $P < 0.05$ ) reduced the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> on day 1 of culture, and of PGF<sub>2α</sub> on day 2 of culture. The output of 6-keto-PGF<sub>1α</sub> was significantly ( $P < 0.05$ ) increased on day 3 of culture by EGTA. TMB-8 (30 and 100 μmol/l) significantly ( $P < 0.05$ ) reduced the output of PGF<sub>2α</sub> on days 1, 2 and 3 of culture. TMB-8 (30 μmol/l) significantly reduced PGE<sub>2</sub> output on day 2 of culture, and 100 μmol TMB-8/l significantly ( $P < 0.05$ ) increased PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs on day 3 of culture.

The output of PGF<sub>2α</sub> was significantly ( $P < 0.05$ ) reduced by TFP (200 μmol/l on day 1 of culture, 100 μmol/l on day 2 of culture and 100 and 200 μmol/l on day 3 of culture). The outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were significantly ( $P < 0.05$ ) increased by TFP (100 and 200 μmol/l) on all 3 days of culture. W-7 (150 and 300 μmol/l) significantly ( $P < 0.05$ ) inhibited PGF<sub>2α</sub> output on all 3 days of culture. The output of

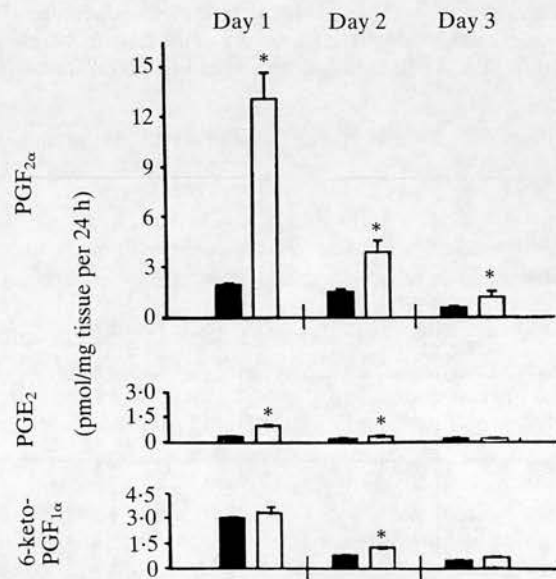


FIGURE 1. Mean ( $\pm$  S.E.M.,  $n = 10$ ) outputs of prostaglandin (PG) F<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from guinea-pig endometrium, removed on day 7 (solid bars) and day 15 (open bars) of the oestrous cycle, on days 1, 2 and 3 of tissue culture.

\*Significantly higher ( $P < 0.05$ ) compared with day 7 value for same PG on same day of culture (Student's *t*-test, or modified *t*-test if variances were unequal by variance ratio *F*-test).

PGE<sub>2</sub> was significantly ( $P < 0.05$ ) increased by W-7 (150 and 300 μmol/l) on all 3 days of culture except at 150 μmol/l on day 1. The output of 6-keto-PGF<sub>1α</sub> was significantly ( $P < 0.05$ ) increased by W-7 (150 and 300 μmol/l) on day 2 of culture, and by W-7 (150 μmol/l) on day 3 of culture.

Nifedipine (1 and 10 μmol/l) had no significant effect on the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> on all 3 days of culture. Nifedipine (100 μmol/l) significantly ( $P < 0.05$ ) inhibited the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> on days 1 and 2 of culture, and PGF<sub>2α</sub> output on day 3 of culture. Verapamil (1 μmol/l) had no effect on the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> on the 3 days of culture. The output of PGF<sub>2α</sub> was significantly ( $P < 0.05$ ) reduced by verapamil (10 and 100 μmol/l) on day 1 of culture, and by verapamil (10 μmol/l) on day 2 of culture. The outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were significantly ( $P < 0.05$ ) increased by verapamil (100 μmol/l) on days 2 and 3 of culture, and 6-keto-PGF<sub>1α</sub> output was significantly ( $P < 0.05$ ) increased by verapamil (50 μmol/l) on day 3 of culture.

The outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day-15 and day-7 endometrium were not affected by TPA (1.6–40.5 nmol/l) on any day of culture.

TABLE 1. Mean ( $\pm$  S.E.M.,  $n=10$ ) output of prostaglandin (PG)  $\text{F}_{2a}$  (expressed as % of day 1 control (C) output) from guinea-pig endometrium removed on day 15 of the oestrous cycle (unless otherwise stated) and cultured in the absence (C) or presence (T) of EGTA, TMB-8, trifluoperazine (TFP), W-7, nifedipine (nifed), verapamil (verap) and phorbol 12-myristate 13-acetate (TPA)

		Output of $\text{PGF}_{2a}$ (% of control on day 1 of culture)					
		Day 1		Day 2		Day 3	
		C	T	C	T	C	T
<b>Treatment</b>							
EGTA	2 mmol/l	100	65.3 $\pm$ 11.2*	40.6 $\pm$ 4.2	24.6 $\pm$ 6.2*	14.3 $\pm$ 1.8	12.1 $\pm$ 2.0
TMB-8	30 $\mu$ mol/l	100	66.3 $\pm$ 5.0*	32.8 $\pm$ 4.2	18.6 $\pm$ 3.7*	8.7 $\pm$ 1.7	4.3 $\pm$ 0.7*
	100 $\mu$ mol/l		56.3 $\pm$ 5.7*		12.2 $\pm$ 1.6*		4.6 $\pm$ 0.8*
TFP	100 $\mu$ mol/l	100	83.2 $\pm$ 8.4	40.8 $\pm$ 5.7	20.4 $\pm$ 3.2*	20.7 $\pm$ 5.5	3.4 $\pm$ 0.7*
	200 $\mu$ mol/l		76.4 $\pm$ 9.4*		27.4 $\pm$ 4.9		4.6 $\pm$ 1.3*
W-7	150 $\mu$ mol/l	100	36.2 $\pm$ 3.1*	34.8 $\pm$ 3.2	13.1 $\pm$ 1.6*	8.8 $\pm$ 1.2	4.1 $\pm$ 0.4*
	300 $\mu$ mol/l		35.9 $\pm$ 3.5*		9.9 $\pm$ 2.2*		1.7 $\pm$ 0.5*
Nifed	1 $\mu$ mol/l	100	111.2 $\pm$ 8.4	29.2 $\pm$ 1.8	34.6 $\pm$ 3.6	9.0 $\pm$ 2.0	12.3 $\pm$ 5.1
	10 $\mu$ mol/l		98.5 $\pm$ 8.4		30.8 $\pm$ 3.5		8.3 $\pm$ 1.4
	100 $\mu$ mol/l		57.5 $\pm$ 1.5*		10.7 $\pm$ 1.8*		3.4 $\pm$ 1.5*
Verap	1 $\mu$ mol/l		96.2 $\pm$ 11.4		26.0 $\pm$ 2.3		8.6 $\pm$ 3.0
	10 $\mu$ mol/l		79.3 $\pm$ 8.0*		23.3 $\pm$ 2.0*		6.0 $\pm$ 0.5
	50 $\mu$ mol/l		82.7 $\pm$ 7.7		25.9 $\pm$ 2.9		9.1 $\pm$ 1.3
	100 $\mu$ mol/l		76.2 $\pm$ 5.1*		35.3 $\pm$ 3.2		9.6 $\pm$ 0.7
TPA	1.6 nmol/l	100	102.2 $\pm$ 10.7	28.4 $\pm$ 3.1	32.3 $\pm$ 5.1	6.8 $\pm$ 1.1	7.0 $\pm$ 1.1
	8.1 nmol/l		98.2 $\pm$ 10.9		33.1 $\pm$ 5.9		7.7 $\pm$ 1.7
	40.5 nmol/l		104.5 $\pm$ 10.2		37.0 $\pm$ 5.6		6.7 $\pm$ 1.7
TPA	1.6 nmol/l	100	107.4 $\pm$ 7.6	70.2 $\pm$ 11.6	75.5 $\pm$ 16.9	29.9 $\pm$ 5.5	27.2 $\pm$ 7.7
Day 7	8.1 nmol/l		108.6 $\pm$ 12.1		73.1 $\pm$ 11.7		31.5 $\pm$ 5.0
	40.5 nmol/l		114.4 $\pm$ 14.7		76.0 $\pm$ 11.2		30.5 $\pm$ 8.2

\*Significantly lower ( $P < 0.05$ ) compared with corresponding control value for the same day (Student's one- or two-tailed  $t$ -test, as appropriate).

### Effect of using $\text{Ca}^{2+}$ -depleted medium

The basal outputs of  $\text{PGF}_{2a}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1a}$  from day-15 guinea-pig endometrium in  $\text{Ca}^{2+}$ -depleted medium compared with  $\text{Ca}^{2+}$ -containing medium are shown in Fig. 2. On days 1 and 2 of tissue culture, the outputs of  $\text{PGF}_{2a}$  and  $\text{PGE}_2$  were significantly ( $P < 0.05$ ) reduced by 48 and 39% respectively in  $\text{Ca}^{2+}$ -depleted medium. The output of 6-keto- $\text{PGF}_{1a}$  was significantly ( $P < 0.05$ ) reduced by 14% in  $\text{Ca}^{2+}$ -depleted medium on day 1 of culture.

The output of  $\text{PGF}_{2a}$  in  $\text{Ca}^{2+}$ -depleted medium was further and significantly ( $P < 0.05$ ) reduced by EGTA (2 mmol/l), TMB-8 (100  $\mu$ mol/l), W-7 (150  $\mu$ mol/l), nifedipine (100  $\mu$ mol/l) and verapamil (100  $\mu$ mol/l) on day 1 of culture, and by EGTA (2 mmol/l), TMB-8 (100  $\mu$ mol/l) and nifedipine (100  $\mu$ mol/l) on day 2 of culture. The output of  $\text{PGE}_2$  in  $\text{Ca}^{2+}$ -depleted medium was significantly ( $P < 0.05$ ) reduced by nifedipine (100  $\mu$ mol/l) and significantly increased by TMB-8 (100  $\mu$ mol/l), W-7 (150  $\mu$ mol/l) and EGTA (100  $\mu$ mol/l) on days 2 or 3 of culture. The output of 6-keto- $\text{PGF}_{1a}$  in  $\text{Ca}^{2+}$ -free medium was significantly ( $P < 0.05$ ) reduced by EGTA (2 mmol/l) and nifedipine (100  $\mu$ mol/l) on day 1 of culture and significantly ( $P < 0.05$ ) stimulated by EGTA (2 mmol/l),

TMB-8 (100  $\mu$ mol/l), W-7 (150  $\mu$ mol/l) and verapamil (100  $\mu$ mol/l) on days 2 and 3 of culture (Table 4).

### DISCUSSION

The basal outputs of  $\text{PGF}_{2a}$  and, to a much lesser extent,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1a}$  from guinea-pig endometrium maintained in culture were higher on day 15 than on day 7 of the oestrous cycle. This shows that a preferential stimulation of endometrial  $\text{PGF}_{2a}$  output occurred *in vivo* between days 7 and 15 of the cycle, and that this stimulation is maintained in tissue culture, particularly during the first 24 h of culture, in the absence of the steroid hormones (i.e. oestradiol and progesterone) responsible for this stimulation (Poyser, 1983a). These findings are in agreement with similar studies performed on the superfused guinea-pig uterus (Poyser & Brydon, 1983).

Prostaglandin output from the superfused day-15 guinea-pig uterus is stimulated further by the calcium ionophore A23187 (Poyser & Brydon, 1983), an action which is dependent upon extracellular  $\text{Ca}^{2+}$  (Poyser, 1984). Raising the intracellular free  $\text{Ca}^{2+}$  concentration therefore stimulates  $\text{PGF}_{2a}$  synthesis



TABLE 2. Mean ( $\pm$  S.E.M.,  $n = 10$  unless otherwise indicated) output of prostaglandin (PG) E<sub>2</sub> (expressed as % of day 1 control (C) output) from guinea-pig endometrium removed on day 15 of the oestrous cycle (unless otherwise stated) and cultured in the absence (C) or presence (T) of EGTA, TMB-8, trifluoperazine (TFP), W-7, nifedipine (nifed), verapamil (verap) and phorbol 12-myristate 13-acetate (TPA)

		Output of PGE <sub>2</sub> (% of control on day 1 of culture)					
		Day 1		Day 2		Day 3	
		C	T	C	T	C	T
<b>Treatment</b>							
EGTA	2 mmol/l	100	47.4 $\pm$ 6.0*	38.0 $\pm$ 3.7	27.2 $\pm$ 5.0	21.0 $\pm$ 4.7	25.1 $\pm$ 7.1
TMB-8	30 $\mu$ mol/l	100	100.1 $\pm$ 9.8	42.4 $\pm$ 5.7	24.7 $\pm$ 4.4*	16.0 $\pm$ 2.9	12.6 $\pm$ 2.5
	100 $\mu$ mol/l		119.9 $\pm$ 13.0		47.5 $\pm$ 6.3		37.5 $\pm$ 6.0
TFP	100 $\mu$ mol/l	100	231.0 $\pm$ 37.7†	48.6 $\pm$ 5.6	204.5 $\pm$ 46.6†	28.7 $\pm$ 5.1	87.5 $\pm$ 15.9†
	200 $\mu$ mol/l		283.6 $\pm$ 42.5†		207.4 $\pm$ 25.3†		108.0 $\pm$ 19.3†
W-7	150 $\mu$ mol/l	100	109.4 $\pm$ 7.3	37.5 $\pm$ 5.9	87.3 $\pm$ 9.5†	16.7 $\pm$ 3.1	46.7 $\pm$ 6.3†
	300 $\mu$ mol/l		180.4 $\pm$ 20.2†		121.3 $\pm$ 20.6†		27.6 $\pm$ 3.1†
Nifed	1 $\mu$ mol/l	100	112.3 $\pm$ 9.8	39.2 $\pm$ 3.0	43.5 $\pm$ 4.7	20.5 $\pm$ 3.3	16.8 $\pm$ 3.8
	10 $\mu$ mol/l		86.1 $\pm$ 7.3		31.5 $\pm$ 4.6		18.2 $\pm$ 3.4
	100 $\mu$ mol/l		48.7 $\pm$ 9.0*		17.1 $\pm$ 4.9*		13.0 $\pm$ 2.7
Verap	1 $\mu$ mol/l		96.2 $\pm$ 9.9		35.7 $\pm$ 4.1		12.0 $\pm$ 3.0
	10 $\mu$ mol/l		88.2 $\pm$ 9.1		32.9 $\pm$ 3.1		15.3 $\pm$ 1.8
	50 $\mu$ mol/l		102.2 $\pm$ 10.5		39.8 $\pm$ 5.8		35.6 $\pm$ 6.9
	100 $\mu$ mol/l		111.2 $\pm$ 10.0		67.5 $\pm$ 7.6†		54.0 $\pm$ 10.9†
TPA	1.6 nmol/l		114.0 $\pm$ 9.4	33.7 $\pm$ 2.2	37.6 $\pm$ 3.5	11.1 $\pm$ 1.8	12.0 $\pm$ 2.2
	8.1 nmol/l		116.8 $\pm$ 10.2		39.8 $\pm$ 4.9		10.5 $\pm$ 1.3
	40.5 nmol/l		106.4 $\pm$ 7.9		41.2 $\pm$ 4.1		8.5 $\pm$ 1.2
TPA	1.6 nmol/l	100	91.0 $\pm$ 8.6	48.6 $\pm$ 16.0†	55.0 $\pm$ 10.2†	25.8 $\pm$ 7.6†	18.4 $\pm$ 11.4†
(Day 7)	8.1 nmol/l		113.9 $\pm$ 15.6		52.4 $\pm$ 10.5†		23.0 $\pm$ 9.7†
	40.5 nmol/l		110.7 $\pm$ 8.9		53.0 $\pm$ 16.2†		26.0 $\pm$ 6.8†

Significantly ( $P < 0.05$ ) \*lower or †higher than corresponding control (C) value for the same day (Student's one- or two-tailed  $t$ -test, as appropriate).

‡Mean ( $\pm$  S.E.M.) of four to six values. Other values were below the limit of detection (0.03 pmol/mg tissue per 24 h), and these values were obtained for control and the corresponding treated samples.

by, and release from, the guinea-pig uterus. However, the high basal output of PGF<sub>2 $\alpha$</sub>  from the day-15 guinea-pig uterus is not reduced during a 2.5 h superfusion with Ca<sup>2+</sup>-free medium (Poyser, 1984), or during a 30-min superfusion with the intracellular calcium antagonist TMB-8 (Poyser, 1985a). These findings raised the question of whether Ca<sup>2+</sup> is necessary for the increase in endometrial PGF<sub>2 $\alpha$</sub>  production which occurs after day 11 of the cycle. The present study showed that EGTA and TMB-8 in Ca<sup>2+</sup>-containing medium significantly reduced basal PGF<sub>2 $\alpha$</sub>  output from day-15 guinea-pig endometrium maintained for 3 days in tissue culture. The use of Ca<sup>2+</sup>-depleted medium had the same effect. TMB-8 and EGTA also caused a further small reduction in endometrial PGF<sub>2 $\alpha$</sub>  output in Ca<sup>2+</sup>-depleted medium, suggesting that EGTA acts intracellularly as well as extracellularly. Alternatively, the inhibitory action of EGTA may be due to it chelating residual Ca<sup>2+</sup> in the Ca<sup>2+</sup>-depleted medium. These findings indicate that Ca<sup>2+</sup>, primarily of extracellular origin, is necessary for the high output of PGF<sub>2 $\alpha$</sub>  from day-15 guinea-pig endometrium. Similar findings have been reported for PGE<sub>2</sub> output from human amnion cells (Olson,

Opavsky & Challis, 1983). However, the possibility cannot be excluded that endometrial PGF<sub>2 $\alpha$</sub>  synthesis is dependent upon the mobilization of Ca<sup>2+</sup> from intracellular stores, and that the extracellular Ca<sup>2+</sup> is necessary solely to replenish these stores. Nevertheless, previous studies (Poyser, 1984, 1985a) have shown that PGF<sub>2 $\alpha$</sub>  output from day-15 guinea-pig uterus superfused for short periods can be maintained in the absence of extracellular and non-bound intracellular Ca<sup>2+</sup>.

The high basal output of PGF<sub>2 $\alpha$</sub>  from day-15 guinea-pig endometrium was reduced by the calmodulin inhibitors W-7 and, to a lesser extent, TFP. These findings are consistent with these compounds preventing A23187-stimulated increases in uterine PGF<sub>2 $\alpha$</sub>  output (Poyser, 1985a,b), and suggest that calmodulin is involved in the process by which an increase in the intracellular free Ca<sup>2+</sup> concentration stimulates PLA<sub>2</sub> activity. However, there is some debate as to whether PLA<sub>2</sub> is a calmodulin-dependent enzyme (Withnall & Brown, 1982; Moskowitz, Andres, Silva *et al.* 1985). The activity of platelet PLA<sub>2</sub> is stimulated by acidic phospholipids (such as phosphatidic acid and phosphatidylserine), but not by calmodulin, in a Ca<sup>2+</sup>-

TABLE 3. Mean ( $\pm$  S.E.M.,  $n=10$ ) output of 6-keto-prostaglandin (PG)  $F_{1\alpha}$  (expressed as % of day 1 control (C) output) from guinea-pig endometrium removed on day 15 of the oestrous cycle (unless otherwise stated) and cultured in the absence (C) or presence (T) of EGTA, TMB-8, trifluoperazine (TFP), W-7, nifedipine (nifed), verapamil (verap) and phorbol 12-myristate 13-acetate (TPA)

		Output of 6-keto-PGF $_{1\alpha}$ (% of control on day 1 of culture)					
		Day 1		Day 2		Day 3	
		C	T	C	T	C	T
<b>Treatment</b>							
EGTA	2 mmol/l	100	72.1 $\pm$ 10.7*	35.3 $\pm$ 3.0	38.0 $\pm$ 6.9	14.9 $\pm$ 1.6	29.1 $\pm$ 4.7†
TMB-8	30 $\mu$ mol/l	100	106.8 $\pm$ 5.5	27.8 $\pm$ 2.7	25.4 $\pm$ 2.1	12.3 $\pm$ 2.2	12.9 $\pm$ 2.5
	100 $\mu$ mol/l		114.7 $\pm$ 8.5		37.1 $\pm$ 4.0		30.9 $\pm$ 3.5†
TFP	100 $\mu$ mol/l	100	163.2 $\pm$ 15.3†	33.3 $\pm$ 3.6	73.9 $\pm$ 10.0†	19.5 $\pm$ 4.4	29.9 $\pm$ 4.9
	200 $\mu$ mol/l		179.2 $\pm$ 20.2†		76.1 $\pm$ 8.4†		36.9 $\pm$ 6.4†
W-7	150 $\mu$ mol/l	100	108.1 $\pm$ 6.8	27.6 $\pm$ 1.8	52.1 $\pm$ 5.5†	14.4 $\pm$ 2.0	40.6 $\pm$ 5.0†
	300 $\mu$ mol/l		134.4 $\pm$ 18.8		53.2 $\pm$ 6.2†		13.4 $\pm$ 3.0
Nifed	1 $\mu$ mol/l	100	99.1 $\pm$ 6.8	27.9 $\pm$ 1.8	29.0 $\pm$ 2.5	14.0 $\pm$ 1.0	12.9 $\pm$ 1.5
	10 $\mu$ mol/l		87.8 $\pm$ 7.2		23.6 $\pm$ 2.5		13.9 $\pm$ 1.7
	100 $\mu$ mol/l		74.4 $\pm$ 4.7*		21.7 $\pm$ 2.2*		13.5 $\pm$ 1.0
Verap	1 $\mu$ mol/l		95.3 $\pm$ 6.3		28.2 $\pm$ 1.7		13.1 $\pm$ 1.2
	10 $\mu$ mol/l		93.6 $\pm$ 9.7		28.9 $\pm$ 2.1		12.6 $\pm$ 1.0
	50 $\mu$ mol/l		104.0 $\pm$ 6.8		28.6 $\pm$ 2.5		19.6 $\pm$ 1.9†
	100 $\mu$ mol/l		112.7 $\pm$ 10.9		36.6 $\pm$ 1.9†		23.0 $\pm$ 2.4†
TPA	1.6 nmol/l	100	106.4 $\pm$ 6.0	31.0 $\pm$ 2.3	30.5 $\pm$ 2.9	10.2 $\pm$ 0.9	11.5 $\pm$ 1.3
	8.1 nmol/l		114.7 $\pm$ 8.3		37.4 $\pm$ 5.3		10.1 $\pm$ 0.9
	40.5 nmol/l		106.6 $\pm$ 8.0		31.7 $\pm$ 3.2		9.8 $\pm$ 0.9
TPA (Day 7)	1.6 nmol/l	100	98.7 $\pm$ 6.4	24.9 $\pm$ 1.7	23.4 $\pm$ 1.9	13.4 $\pm$ 1.4	12.4 $\pm$ 1.1
	8.1 nmol/l		105.2 $\pm$ 10.6		37.4 $\pm$ 5.3		15.2 $\pm$ 2.1
	40.5 nmol/l		107.8 $\pm$ 4.0		31.7 $\pm$ 3.2		13.8 $\pm$ 1.6

Significantly ( $P < 0.05$ ) \*lower or †higher than corresponding control value for the same day (Student's one- or two-tailed  $t$ -test, as appropriate).

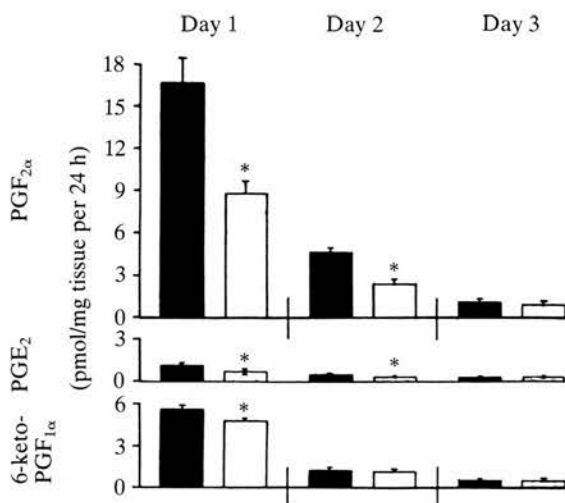


FIGURE 2. Mean ( $\pm$  S.E.M.,  $n=10$ ) outputs of prostaglandin (PG)  $F_{2\alpha}$ ,  $PGE_2$  and 6-keto-PGF $_{1\alpha}$  from guinea-pig endometrium, removed on day 15 of the oestrous cycle, in the presence (solid bars) and absence (open bars) of  $Ca^{2+}$  on days 1, 2 and 3 of culture. \*Significantly lower ( $P < 0.05$ ) than the value in the presence of  $Ca^{2+}$  for the same PG on the same day of culture (Student's  $t$ -test).

dependent manner. The calmodulin inhibitors TFP and W-7 inhibit this stimulation of  $PLA_2$  by preventing the binding of  $Ca^{2+}$  to the acidic phospholipids (Watanabe, Hashimoto, Teramoto *et al.* 1986). Whether calmodulin or acidic phospholipids mediate the intracellular action of  $Ca^{2+}$  in guinea-pig endometrium merits further investigation, but the reduction in  $PGF_{2\alpha}$  output from day-15 guinea-pig endometrium by TFP and W-7 is further evidence that  $Ca^{2+}$  is necessary for increased  $PGF_{2\alpha}$  production by the guinea-pig uterus from day 11 of the cycle.

Nifedipine in concentrations (1 and 10  $\mu$ mol/l) which normally block calcium channels had no effect on endometrial  $PGF_{2\alpha}$  output. Verapamil (1  $\mu$ mol/l) also had no effect. However, nifedipine (100  $\mu$ mol/l) had a marked inhibitory effect on  $PGF_{2\alpha}$  output, whereas verapamil (10–100  $\mu$ mol/l) had a small, sometimes significant, inhibitory action on  $PGF_{2\alpha}$  production. The inhibitory effect of both drugs was still present in  $Ca^{2+}$ -depleted medium. Nifedipine and the verapamil derivative, D-600, inhibit calmodulin (Andersson, Drakenberg, Thulin & Forsen, 1983; Minocherhomjee & Roufogalis, 1984), so nifedipine and verapamil may be inhibiting  $PGF_{2\alpha}$  synthesis in a manner similar to that of TFP and W-7. Also, nifedipine (100  $\mu$ mol/l) and verapamil (100  $\mu$ mol/l) inhibit  $PGI_2$  output from rabbit mesothelial cells (Van de

TABLE 4. Mean ( $\pm$ S.E.M.,  $n=10$ ) output of prostaglandin (PG) F<sub>2a</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> (expressed as % of day 1 control (C) output for the same PG) from guinea-pig endometrium removed on day 15 of the oestrous cycle and cultured in the absence (C) or presence (T) of EGTA, TMB-8, W-7, nifedipine (nifed) and verapamil (verap) using Ca<sup>2+</sup>-depleted medium

PG and treatment	Output of PGs (% of control on day 1 of culture)					
	Day 1		Day 2		Day 3	
	C	T	C	T	C	T
<b>PGF<sub>2a</sub></b>						
2 mmol/l EGTA	100	70.4 $\pm$ 10.9*	26.8 $\pm$ 1.4	15.8 $\pm$ 1.5*	11.0 $\pm$ 0.9	8.8 $\pm$ 0.7
100 $\mu$ mol/l TMB-8		82.5 $\pm$ 6.7*		20.4 $\pm$ 1.9*		6.8 $\pm$ 1.0
150 $\mu$ mol/l W-7		71.2 $\pm$ 6.6*		30.5 $\pm$ 3.8		9.1 $\pm$ 1.5
100 $\mu$ mol/l nifed		66.3 $\pm$ 7.6*		15.5 $\pm$ 1.7*		9.4 $\pm$ 1.2
100 $\mu$ mol/l verap		82.6 $\pm$ 7.1*		33.7 $\pm$ 3.5		14.5 $\pm$ 2.6
<b>PGE<sub>2</sub></b>						
2 mmol/l EGTA	100	88.5 $\pm$ 7.9	36.3 $\pm$ 4.4	42.1 $\pm$ 3.6	22.8 $\pm$ 4.5	33.7 $\pm$ 4.7
100 $\mu$ mol/l TMB-8		124.8 $\pm$ 9.1†		59.5 $\pm$ 7.3†		36.9 $\pm$ 5.6
150 $\mu$ mol/l W-7		135.9 $\pm$ 13.2†		111.3 $\pm$ 19.2†		53.9 $\pm$ 9.4†
100 $\mu$ mol/l nifed		22.0 $\pm$ 4.0*		12.6 $\pm$ 3.2*		7.7 $\pm$ 1.8*
100 $\mu$ mol/l verap		106.5 $\pm$ 3.6		71.2 $\pm$ 10.3		47.5 $\pm$ 10.0†
<b>6-keto-PGF<sub>1a</sub></b>						
2 mmol/l EGTA	100	84.7 $\pm$ 5.7*	24.5 $\pm$ 1.8	62.9 $\pm$ 4.0†	11.9 $\pm$ 1.1	37.9 $\pm$ 3.0†
100 $\mu$ mol/l TMB-8		94.9 $\pm$ 6.2		36.2 $\pm$ 2.7†		20.5 $\pm$ 2.0†
150 $\mu$ mol/l W-7		108.6 $\pm$ 5.3		44.1 $\pm$ 4.2†		24.6 $\pm$ 3.6†
100 $\mu$ mol/l nifed		83.4 $\pm$ 6.4*		24.5 $\pm$ 2.3		13.1 $\pm$ 1.5
100 $\mu$ mol/l verap		103.9 $\pm$ 6.5		35.5 $\pm$ 3.6†		20.1 $\pm$ 3.8†

Significantly ( $P < 0.05$ ) \*lower or †higher than corresponding control value for the same PG on the same day (Student's one- or two-tailed  $t$ -test, as appropriate).

Velde, Van den Bossche, Bult & Herman, 1986). It is concluded that extracellular Ca<sup>2+</sup> required for the stimulation of PLA<sub>2</sub> activity, and hence PGF<sub>2a</sub> synthesis, does not enter the endometrium by voltage-dependent channels, and that the inhibitory effect of nifedipine and verapamil on endometrial PGF<sub>2a</sub> synthesis is due to an intracellular action.

The inhibitory effects of removal of extracellular Ca<sup>2+</sup> on outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> from the day-15 guinea-pig endometrium in culture were less marked than on PGF<sub>2a</sub> output. This is probably due to the outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> being lower than the output of PGF<sub>2a</sub>, particularly at the start of the culture period (i.e. compared with day-7 endometrium, the basal outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> from day-15 endometrium had not been stimulated to the same extent as the basal output of PGF<sub>2a</sub> and thus there was less scope for inhibition to occur). Paradoxically, TMB-8, TFP, W-7, verapamil and, to a lesser extent, EGTA stimulated the outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub>. Previous studies have shown that while TMB-8, TFP and W-7 prevent the increases in PG output from the superfused guinea-pig uterus induced by A23187, on their own these compounds cause a small stimulation in the outputs of PGF<sub>2a</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> (Poyser, 1985a,b). Thus TMB-8, TFP and W-7 have opposing actions as regards PG production. In the present study, it is clear that the inhibitory effect predominates as regards PGF<sub>2a</sub> output, and that the stimulatory effect predominates as regards

PGE<sub>2</sub> and 6-keto-PGF<sub>1a</sub> outputs. Verapamil and EGTA, but not nifedipine, have similar opposing actions. The stimulatory effect is present in Ca<sup>2+</sup>-free medium suggesting that it is an intracellular process, possibly due to a direct interaction with the membranes of the endoplasmic reticulum, the main site of PLA<sub>2</sub> and PG-synthesizing enzymes (DeWitt, Rollins, Day *et al.* 1981; Lagarde, Menashi & Crawford, 1981; Carey, Menashi & Crawford, 1982). Trifluoperazine had a greater stimulatory effect on PG output than the other compounds, which probably accounts for its weaker inhibitory effect on PGF<sub>2a</sub> output.

In platelets, protein kinase C catalyses phosphorylation of a lipocortin-like protein thus rendering it inactive (Touqui, Rothhut, Shaw *et al.* 1986), and thereby potentiates platelet thromboxane synthesis, in response to appropriate stimuli (Mobley & Tai, 1985), due to the consequent enhancement of PLA<sub>2</sub> activity. TPA, an activator of protein kinase C, had no effect on PGF<sub>2a</sub> production by day-7 and day-15 guinea-pig endometrium. Protein kinase C is normally activated by diglyceride formed by the action of phospholipase C (PLC) on phosphatidylinositol (PI) or polyphosphoinositides (PIP and PIP<sub>2</sub>; see Berridge & Irvine, 1984). The present findings are consistent with a previous study (Ning & Poyser, 1984) which showed that the turnovers of PI, PIP and PIP<sub>2</sub> in guinea-pig endometrium were not increased on day 15 (a day of stimulated endometrial PGF<sub>2a</sub> output) compared with day



7 (a day of low endometrial  $PGF_{2\alpha}$  output) of the cycle. Thus, these investigations show that there is no stimulation of the PI cycle at the time of increased  $PGF_{2\alpha}$  synthesis in the guinea-pig endometrium, and that endometrial  $PLA_2$  activity in the guinea-pig is not normally restrained by a protein kinase C-inactivated lipocortin. TPA stimulates  $PGF_{2\alpha}$  synthesis by cultured human endometrial cells (Skinner, Liggins, Wilson & Neale, 1984), indicating that differences exist in the intracellular mechanisms controlling  $PGF_{2\alpha}$  synthesis by human and guinea-pig endometrium.

Overall, these studies indicate that  $Ca^{2+}$ , mainly of extracellular origin, is necessary for increased  $PGF_{2\alpha}$  production by the guinea-pig endometrium from day 11 of the oestrous cycle. A previous study has shown that this increased output of  $PGF_{2\alpha}$  is prevented by the protein synthesis inhibitor, actinomycin D (Poyser & Riley, 1985, 1987). We propose, therefore, that oestradiol stimulates the synthesis of a protein which, on a progesterone-primed uterus, causes an increase in the endometrial free intracellular  $Ca^{2+}$  concentration and thereby activates  $PLA_2$  to release arachidonic acid from phospholipids for  $PGF_{2\alpha}$  synthesis. The release of arachidonic acid for  $PGE_2$  synthesis by transformed mouse fibroblasts in response to serum, thrombin and bradykinin (Pong, Hong & Levine, 1977), for  $PGE_2$  synthesis by skeletal muscle (Turinsky, 1985), and for the stimulation of  $PGI_2$  synthesis in endothelial cells by bradykinin and leukotrienes (Clark, Littlejohn, Mong & Crooke, 1986), is also dependent upon increased protein synthesis. Furthermore, increased  $PGI_2$  production in endothelial cells by bradykinin is dependent upon extracellular  $Ca^{2+}$ , but is not dependent upon the opening of voltage-dependent  $Ca^{2+}$  channels (Whorton, Willis, Kent & Young, 1984). Our findings using the guinea-pig endometrium are therefore consistent with findings in other tissues that the release of arachidonic acid, the rate-limiting step for PG synthesis (Vogt, 1978), is dependent upon increased protein synthesis and extracellular  $Ca^{2+}$ .

## ACKNOWLEDGEMENTS

This study was supported by a grant from the SERC, and an MRC Research Scholarship to S.C.R. The technical assistance of Miss L. Marshall is much appreciated. Authentic prostaglandins were kindly supplied by the Upjohn Co., Kalamazoo, MI, U.S.A.

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